

**Studies on parapoxvirus antigens through the
development of monoclonal antibodies to orf virus**

Thesis submitted for the Degree of Doctor of Philosophy

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December 1997



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ABSTRACT

Orf virus is a worldwide cause of a significant disease of sheep and goats, and in many countries there is a need to differentiate orf virus infections from those caused by other poxviruses. Twenty-five monoclonal antibodies (mabs) against orf virus, a parapoxvirus (ppv), were produced following the immunisation of mice with a lysate of cells infected with orf -11 virus. These mabs, together with 2 others recloned from an earlier fusion, were identified by ELISA and IFT and characterised. No neutralising activity was shown by any of the mabs. The size of orf proteins detected by the mabs was measured using western blotting and radioimmunoprecipitation (RIP). Western blotting was conducted with two types of orf-11 antigen preparations:- gradient purified virus or a lysate of orf-11 infected cells. Five mabs detected a protein of approximately 40 kDa with both purified virus and infected lysate antigens. Three mabs detected a protein approximately 65 kDa in size, but only with infected cell lysate antigen. In RIP studies, 21 mabs produced bands 13 of which were against the 65 kDa protein, 7 against the 40 kDa protein while one was against a 50 kDa protein. Twenty-one of the 27 mabs reacted with at least two of 18 vaccinia virus orf virus (VVOV) recombinants expressing a library of orf genome fragments of the NZ-2 virus strain. Four of the mabs which had identified the native 40kDa protein reacted with 2 overlapping recombinants (245 and 247). Seventeen of the mabs, 16 of which had identified the native 65 kDa protein recognised three recombinants 79, 285 and 286 all of which contain different inserts from the same region of the orf virus genome. Subsequent sequencing of the overlapping site between recombinants 245 and 247 by New Zealand collaborators has identified a new orf gene, designated F1L which has been shown to be analogous to the H3L vaccinia virus gene which codes for an immunodominant 35 kDa envelope protein. Cells infected with a new VVOV recombinant expressing only the F1L orf gene showed positive fluorescence with 3 of the 4 mabs which reacted with the 245 and 247 recombinants, confirming the target of these mabs is the product of the F1L gene.

Cross-reactivity of the 27 mabs against eight parapoxviruses (ppvs) clearly differentiated the 6 ruminant ppvs from ppvs isolated from a seal and a squirrel. In IFT, 11 mabs reacted with the 4 sheep and 2 cattle ppvs; seven mabs recognised the 4 sheep and 1 or other of the cattle ppvs; four mabs detected the 6 ruminant ppvs and the seal ppv; two mabs reacted with the sheep isolates only; one reacted with the 6 ruminant ppvs and the squirrel ppv and one mab

(1C7) detected all 8 ppvs. None of the mabs reacted with vaccinia virus but one of the 27, 1C7 has been shown to react with capripoxvirus. The panel of mabs has therefore, demonstrated its ability to discriminate isolates of ruminant ppvs from each other and from capripoxvirus.

On the basis of their protein reactivity and relatively high ELISA ODs, eight mabs were chosen for competition studies with a view to developing a capture ELISA for the rapid detection of orf virus from clinical specimens. The 4 selected mabs against the 40 kDa protein recognised 3 epitopes as did the 4 selected mabs against the 65 kDa protein. A double mab ELISA for detecting orf virus was developed and evaluated using suspensions of cell culture grown virus, detergent-treated infected cell cultures, virus purified from skin scabs and crude detergent-treated skin scabs. The ELISA detected as few as 100 TCID₅₀ of cell culture grown virus and was reproducible for all the orf virus preparations except the crude detergent-treated skin scab. An ELISA which detects orf virus in crude scabs was developed using a polyclonal antibody capture phase and a mab detector which after further optimisation may be of advantage in field situations.

DECLARATION

I declare that all the work presented in this thesis was prepared by myself. Wherever contributions from colleagues has been included this has been fully acknowledged.

Fadhel M T Housawi

DEDICATION

I would like to dedicate this thesis with love to my mother and the rest of my family.

ACKNOWLEDGEMENTS

I would like to acknowledge and thank my supervisor at Moredun, Dr Peter Nettleton for his close supervision and excellent guidance and my University supervisor Dr Keith Sumption for his generous and fruitful advice, encouragement and interest.

I am deeply in debt to Mrs Janice Gilray from whom I have learnt so much, especially how to handle, produce and characterise monoclonal antibodies. Mrs Tracey Fitzgerald for the big effort she has had in trying to sequence and express the 65kDa gene and for other technical help.

I am very grateful to Dr Hugh Reid for his interest, advice and useful discussions. Thanks are also extended to Dr Colin McInnes and Dr David Haig for their encouragement and advice.

I would like to thank Dr Ian McKendrick the statistician at Moredun Research Institute for all his help in statistical work; Mrs Christine Curran and Ms Lynda Ralston for the big effort in typing the thesis and also Mr Brian Easter for the photography.

Thanks are also extended to Dr Andy Mercer and Ms Georgina Roberts, University of Otago, New Zealand for their collaboration and also to Dr Debbie Ireland, Institute of Animal Health, Pirbright Laboratory for screening the mabs against capripoxvirus.

Finally, I am very thankful for all the technical assistance I have received from the staff at Moredun Research Institute:- Dr Gary Entrican; Aileen Edwards; Irene Pow; Iris Campbell; Neil Inglis; Kay Hall; Kay Quinn; Diane Donaldson; Heather Edgar; David Harkins; Stuart Martin and Dr Stephen Fleming.

ABBREVIATIONS USED IN TEXT

BSA	=	Bovine serum albumin
BPSV	=	Bovine papular stomatitis virus
°C	=	Degrees centigrade
CFT	=	Complement fixation test
cm	=	Centimetre(s)
CPE	=	Cytopathic effect
CPV	=	Capripoxvirus
DNA	=	Deoxyribonucleic acid
ELISA	=	Enzyme linked immunosorbent assay
FBS	=	Foetal bovine serum
FITC	=	Fluorescein isothiocyanate
FLM	=	Foetal lamb muscle
g	=	gram(s)
G+C	=	Guanine + Cytosine
GM-CSF	=	Granulocyte macrophage - colony stimulating factor
hr	=	Hour(s)
HRP	=	Horseradish peroxidase
IgA	=	Immunoglobulin A
IgG	=	Immunoglobulin G
IgM	=	Immunoglobulin M
IL	=	Interleukins
ITR	=	Inverted terminal repeat
kDa	=	Kilo Dalton(s)
Kb	=	1000 base pairs
2ME	=	2-mercaptoethanol
mm	=	millimetre(s)
MHC	=	Major histocompatibility complex
μ	=	micron(s)
μg	=	microgram(s)
MNV	=	Milker's node virus
nm	=	nanometre(s)
NP40	=	Nonidet p40
NZ2	=	New Zealand orf virus strain 2
OPD	=	Ortho-phenylene diamine
ORF	=	Open reading frame
OV	=	Orf virus
PAGE	=	Polyacrylamide gel electrophoresis
PBS	=	Phosphate buffered saline
PPV	=	Parapoxvirus
RIP	=	Radioimmunoprecipitation
RNA	=	Ribonucleic acid
SAPU	=	Scottish antibody production unit
SDS	=	Sodium dodecyl sulphate
SPF	=	Specific pathogen free
SPV	=	Swinepoxvirus
TCID ₅₀	=	50% Tissue culture infectivity dose
UV	=	Ultraviolet
VEGF	=	Vascular endothelial growth factor
VPM20	=	Antipestivirus monoclonal antibody
VV	=	Vaccinia virus
VVOV	=	Vaccinia virus orf virus recombinant(s)

CHAPTER 1
REVIEW OF THE LITERATURE

1.1 HISTORY

A disease resembling orf has been recognised and described since the 18th century. In 1856 Youatt reported “black-muzzle” as a disease known to shepherds in Europe. He noted that the malady occurred as a pimples or scabbed eruption around the mouth of the affected sheep and sometimes appeared around the eyes and ears. The disease attacked frequently sucking lambs, and was also seen in full-grown sheep. This is a good description of the disease seen today.

The term orf was first referred to as originating in the Northern and Border counties of Scotland, and described as a contagious dermatitis which affected the feet, legs and face of sheep (Walley, 1890). In a previous report the same author had called the disease pustular fever or contagious ecthyma and preferred not to call it Malignant Aphtha (Walley, 1888). Berry (1901) described a disease that produced lesions involving the mouth, nostrils and coronet of sheep and designated it contagious pustular dermatitis, hair and hoof disease, or “mouth and foot disease”. Glover (1928) noted that orf lesions are usually localised to the external part of the lips, particularly the commissures and nostrils and called it contagious pustular stomatitis, contagious pustular dermatitis (CPD) and Malignant Aphtha.

Despite early awareness of the disease the real cause of the malady was not obvious to early workers. Hoare (1913) was of the opinion that the bacterium *Bacillus necrosis* (*Fusiformis necrophorus*) was the causative organism of the disease. A filterable virus was later shown to be the cause and the experimentally produced pathological stages of the disease in sheep from macule, papule, vesicle and pustule through to crusts were described (Aynaud, 1923). The disease was also reproduced experimentally in goats (Glover, 1928).

Early attempts to transmit the disease to rabbits failed (Aynaud, 1923; Glover, 1928; Howarth, 1929). Only Lanfranchi (1925) claimed success.

Between 1920 and 1930 the malady was being recognised in sheep and goats around the world. Zeller (1920) and Theiler (1928) had reported contagious ecthyma in South Africa. Blanc, Melanidi and Caminopetros (1922) noted CPD in goats in Greece. Following Aynaud's studies, Moussu (1923) investigated more CPD outbreaks in France. Lanfranchi (1925) described the disease in sheep in Italy. Howarth (1929) reported CPD in sheep and goats in California and Schmidt and Tunnicliff (1929) in Texas. In Australia, Seddon and Belschner (1929) noted orf in sheep and goats. Ever since these reports the disease is believed to occur wherever sheep and goats are reared.

1.2 NOMENCLATURE

Orf is a disease which has many synonyms throughout the world. More than 80 have been compiled (Mayr and Buttner, 1990). In Britain the disease is known as orf or CPD (Glover, 1928; Watt, 1982; McKeever, 1984). In America it is called soremouth, contagious ecthyma or "doby mouth" (Howarth, 1929; Schmidt and Hardy, 1932; Boughton and Hardy, 1935). In France it is described as contagious ecthyma or CPD (Aynaud, 1923; Moussu, 1923). In New Zealand and Australia it is recognised as scabbymouth (Seddon and Belschner, 1929; Ohman, 1941; Larsen, 1985).

It is difficult to trace the exact meaning of the word orf. Both modern and old English dictionaries attributed the word orf as being derived from an Anglo-Saxon word for cattle, but this has nothing to do with the description of the disease in sheep. The English dialect dictionary, describes orf as a puny creature, the word being used in the Lothians of Scotland (Robinson and Balassu, 1981). In the Scottish National dictionary the word is defined as a skin disease in sheep or people also called CPD (Grant, 1965).

Several words are reported to have been linked to the word orf. In Yorkshire dialects the word orf has been found to have the same meaning as urf (Grant, 1965). However, orf also has been stated to mean a person in poor condition, a dirty

insignificant person or a dwarfish person (Robinson and Balassu, 1981). Nagington *et al.*, (1965) are of the opinion that orf originated from hrufa which refers to scabby, rough and rugged; it is derived from the old Icelandic.

1.3 THE DISEASE

1.3.1 GENERAL

Orf is a specific contagious disease of sheep and goats, characterised by the formation of macules, papules, vesicles, pustules and scabs on the skin of the lips and nostrils. Lesions may extend to other parts of the body (Trueblood and Chow, 1963). It has been found that orf may occur either in a mild form in which the lesions are strictly localised to the mouth and surrounding tissue or an acute form involving the mucous membranes of the buccal cavity or vulva, and/or the udder (Aynaud, 1923; Glover, 1928; Darbyshire, 1961). Ohman (1941) reported an unusual syndrome with lesions mainly on the scrotum and lips of two-tooth rams. Lateral and posterior sites of the scrotum were covered by the scabs, but no abnormalities were detected in the testicles.

A venereal disease resembling orf has been reported by several workers (Trueblood and Chow, 1963; Watt, 1982; Greig, 1983; Reid, 1991). They described ulcers on the vulva of ewes, and the prepuce and penis of rams. Nevertheless, none of them demonstrated orf virus in the lesions or detected orf virus in specimens from the organs.

The incubation period of orf is three to four days (Greig, 1956; Housawi *et al.*, 1993; Zamri-Saad *et al.*, 1993) and the course of the disease ranges from four to six weeks (Howarth, 1929; Hart *et al.*, 1949; Zamri-Saad *et al.*, 1993). In uncomplicated cases the disease is self-limiting and afebrile. Scabs fall off the skin within six weeks, leaving no scar (Robinson and Balassu, 1981). The morbidity rate is commonly high (70-100%) but the mortality rate in uncomplicated cases is usually not more than 1% (Glover, 1928). High mortality rates between 5-50% have been reported (Aynaud, 1923; Jacotot, 1924; Darbyshire, 1961; Housawi *et al.*, 1991; Radostits *et al.*, 1994).

In one highly devastating outbreak observed in goats by Mazur and Machado (1989) mortality was as high as 93% among kids. Higher mortality rates are believed to be caused by secondary complications. Secondary micro-organisms reported to have caused complications have included *Fusobacterium necrophorum*, (Newsom and Cross, 1934a,b; Watt, 1982), *Dermatophilus congolensis* (Munz, 1969; Watt, 1982), *Streptococcus albus* (Howarth, 1929; Allworth *et al.*, 1987) and screw-worm fly larvae *Cochliomyia americana* (Boughton and Hardy, 1935).

Orf virus gains entry to the body of the animals through injuries to the skin and contact with affected animals or infective materials. Youatt (1856) and Radostits *et al* (1994) are of the opinion that natural infections occur as a result of skin injuries induced by prickly plants or stubble. Once orf has appeared in a flock it spreads very quickly between the animals and animals recovered from the infection produce some immunity against further attacks (Glover, 1928).

1.3.2 LESIONS AND HISTOPATHOLOGY OF THE LESIONS

1.3.2.a Gross Lesion

Orf lesions develop through the typical phases of a pox virus infection, and tend to be proliferative. Orf virus is highly epitheliotropic and produces numerous irregular and confluent lesions on the lips and nostrils of sheep and goats (Berry, 1901; Hoare, 1913; Glover, 1928; Theiler, 1929; Howarth 1929; Samuel *et al.*, 1975; Greig *et al.*, 1984; Concha-Bermejillo, 1993). Lesions start with a reddening and elevation of the skin, followed by papular and vesicular stages, with skin around the vesicles being hyperaemic. Vesicles increase in size, become purulent and subsequently develop into pustules, which rupture and finally develop into scabs (Howarth, 1929). In severe cases lesions may extend to involve the tongue, dental pad and gums (Aynaud, 1923; Glover, 1928; Howarth, 1929; Samuel *et al.*, 1975; Watt, 1982), the coronets, thighs and udder (Glover, 1928; Schmidt and Hardy, 1932; Robinson and Balassu, 1981).

Orf lesions in the buccal cavity don't form scabs, but consist of raised, reddened or greyish areas surrounded by intense hyperaemia (Samuel *et al.*, 1975; Watt, 1982). Newsom and Cross (1931) described the occurrence of orf ulcerative lesions in omasum, mucous surface of the reticulum, rumen and sometimes in the small intestine of the affected animals but they did not demonstrate virus in these lesions. In another severe outbreak in sheep lesions were also reported to involve the gastrointestinal tract, lungs, heart and liver. A suspension of intestinal ulcer from an affected sheep scarified on to the skin of a susceptible lamb produced orf (Darbyshire, 1961). Thiel and Rudolph (1981) described severe orf lesions in a flock of sheep which involved the mouth, tongue and erosions in the rumen. They demonstrated typical orf virus by electron microscopy in lip lesions.

Rubbing off or removing of the scab leads to ulcerative and suppurating wounds, which will cause delay in the healing (Berry, 1901).

1.3.2b Histology

Orf lesions histologically can be divided into three stages. The first stage, papule-vesicle, is characterised by proliferation of the *rete malpighii* (stratum germinativum), the upper cell layers of which become elongated, swollen, and rounded. The cell nuclei shrink and vacuoles appear in various sizes and shapes in the cytoplasm. Vesicles develop in the superficial cells underneath the stratum lucidum with gathering of neutrophils in this region. The second phase, vesicopustule, is characterised by development of an extensive zone of vacuolated cells arising from the proliferation of the *rete malpighii*. Cells degenerate more and more in this site and form an irregular network resemble the "ballonisante stage" of vaccinia lesions. Migration of leukocytes into the area continues until the vesicle changes to a pustule of cell debris. In the third phase, the pustules increase in size and subsequently break through the stratum lucidum. Liberated cell debris will form the crusts. At the end, the leukocytes are completely absorbed, leaving either a fibro-cellular stroma invaded by an irregular mass of epithelial cells or connective tissue

occurs in islands surrounded by epithelial cells, (Glover, 1928; Robinson and Balassu, 1981; McKeever, 1984; Hooser *et al.*, 1989).

A very detailed study of the timing of experimental histological orf change in infected animals was made by Wheeler and Cawley (1955), who found that up to the fourth day post-infection no change was detected in the epidermis. By the fifth and sixth days the epidermis was slightly acanthotic and the cells were undergoing degenerative change (ballooning) and infiltration of the dermis. Other authors obtained the same results (Aynaud, 1923; Selbie, 1944; Lance *et al.*, 1983; Housawi *et al.*, 1993; Zamri-Saad *et al.*, 1993). On the seventh and eighth days degenerative change of the epidermis continued and the remaining cell walls appeared as “a basket weave shape”. Between the 11th and 17th days the surface of the lesions became covered with an intense layer of hyperkeratotic and parakeratotic material resulting from disintegration of the cells of the pustule. The epidermis showed massive pseudoepitheliomatous hyperplasia. Between the 17th and 22nd days the *rete malpighii* grew downward and the dermal papillae grew upward to produce a finger-like papillomatous appearance. Others have described similar findings (Lance, 1983; Jones *et al.*, 1996; Housawi *et al.*, 1993). Finally, from the 22nd to 40th days the papillomatous character gradually disappeared and the lesion gradually involuted. The dermal cellular infiltration resolved and the crust separated.

Cytoplasmic inclusion bodies have been described as a pathological feature of orf virus infection by several authors (Abdussalam, 1957a; Kluge *et al.*, 1972; Samuel *et al.*, 1975; Hooser *et al.*, 1989; Hawkins *et al.*, 1991). Occurrence of the cytoplasmic inclusion bodies is transitory and may not be a reliable diagnostic feature (Jones *et al.*, 1996).

1.3.3 DIAGNOSIS

The diagnosis of orf in sheep and goats is mainly based on the clinical history and the appearance of papules, pustules or scabs on the lips, gums, nostrils, ears, udder, tail and coronets of affected animals. Experimental transmission of the disease to other

sheep and goats can be used to confirm the diagnosis (Berry, 1901; Howarth, 1929; Hart *et al.*, 1949; Connell, 1954; Samuel *et al.*, 1975; Lance *et al.*, 1983; McKeever, 1984; Ndikuwera *et al.*, 1992; Radostits *et al.*, 1994).

To exclude the possibility of other diseases which may produce similar clinical lesions in sheep and goats, differential diagnosis is required between orf, sheep and goat pox, foot-and-mouth disease, bluetongue and occasionally footrot (Mayr and Buttner, 1990; Hawkins *et al.*, 1991).

Because of the unique shape of parapoxviruses, electron microscopy (EM) is a successful and commonly used method for the rapid demonstration of orf-like virus particles (Nagington and Whittle, 1961; Samuel *et al.*, 1975; McKeever, 1984).

The definitive diagnosis of the presence of orf virus in scabs, however, requires serological confirmation. The agar gel precipitation test (AGPT) has been used for the identification of orf antigen (Sharma and Bhatia, 1959; Papadopoulos *et al.*, 1968; Romero-Mercado *et al.*, 1973b; Housawi *et al.*, 1991). The complement fixation test (CFT) was found to be more sensitive and reliable than the AGPT (Romero-Mercado *et al.*, 1973b; Watt, 1982). Orf virus can be difficult to grow *in vitro*, but further confirmation may be achieved by growing the virus in ovine cell cultures such as foetal lamb testes and employing a fluorescent antibody test to detect viral antigen (Kluge *et al.*, 1972; Erickson *et al.*, 1975; Pospischil and Bachman, 1980; Ames *et al.*, 1984; Chubb and Couch, 1985).

Restriction endonuclease profiles were found to be useful for the epidemiological study of orf by purifying virus from scabs of naturally infected and vaccinated animals and extracting DNA (Mazur *et al.*, 1991).

1.3.4 EPIDEMIOLOGY

Early studies showed that orf virus was very resistant and was present in high titre in scabs shed by affected animals (Aynaud, 1923; Boughton and Hardy, 1935). This led

to the suggestion that fallen scabs on the ground are the source of virus for new outbreaks (Theiler, 1928; Glover, 1928; Boughton and Hardy, 1935; Buxton and Fraser, 1977; Watt, 1982). Virus from scabs shed following vaccination is believed to contribute to the environmental pool. This traditional epidemiological view has been questioned by Romero-Mercado *et al.*, (1973a,b), who had difficulty in finding virus in scabs obtained late in the course of the disease.

Robinson and Balassu (1981) stated that carrier animals and latently infected ones are possible sources of new orf outbreaks, but evidence to support this is limited. The possible activation of cryptic virus in ewes has been suggested by the observation of pre-parturition increases in antibody titres (Scott and Maeda, 1978) and the recovery of virus from clinical orf lesions in breeding ewes from which the virus had been isolated when they were lambs (Scott and Osman, 1974). More recently the apparent transmission of orf from clinically normal ewes to orf-naïve sheep has been described (Nettleton *et al.*, 1996). All these observations are of important epidemiological significance since they provide evidence of carrier sheep contributing to the transmission of orf.

Chronic orf cases have been described by several authors. Some of the affected animals were reported to have had the infection for up to eight or nine months (McKeever, 1984; Greig *et al.*, 1984; McKeever *et al.*, 1986; Hooser *et al.*, 1989; Ndikuwera *et al.*, 1992). Persistent poll orf lesions are more commonly seen in rams (Lewis, 1996).

Occurrence of orf lesions on an animal in a flock will soon be followed by appearance of the disease on others. Few animals escape contracting the malady. Spread of the disease depends on direct physical contact with an infected animal or active scabs. This is of importance in housed lambs in particular and provision of solid divisions between groups of lambs is recommended as a prophylactic measure (Robinson and Balassu, 1981; Watt, 1982).

Orf outbreaks can occur at any time of the year but are commonly observed when new generations of lambs and kids are born which is mainly in the spring and summer seasons (Moussu, 1923; Howarth, 1929; Selbie, 1944). In the hot climate of Saudi Arabia, however it has been noted that orf outbreaks occur mainly in the winter and spring (Housawi *et al.*, 1991).

An outbreak of orf has been observed in lambs following ear tagging (Allworth *et al.*, 1987). Orf lesions have also been described on newly ear-tagged goats introduced to experimentally infected animals (Housawi and Abu Elzein, 1991). These two reports suggest that ear tagging of animals should be avoided when there is any evidence of orf in the flock.

1.3.5 HOST RANGE

1.3.5a Natural infection in animals

Although orf has been mainly observed in domestic sheep and goats, it has also been reported to naturally infect other animals. Connell (1954) and Robinson and Balassu (1981), described orf in rocky mountain bighorn sheep (*Ovis canadensis*), while it has also been reported in a mountain goat (*Oreamnos americanus*) (Samuel *et al.*, 1975). Wilkinson and Prydie (1970), reported an unusual orf outbreak in dogs in which the orf lesions were in the form of circular areas of moist dermatitis with ulceration and scab formation. It is believed that the dogs, which were foxhounds, got the infection after being fed on unskinned infected sheep carcasses. Other animals reported to have had natural orf infections are musk oxen (*Ovibos moschatus*) (Mathiesen *et al.*, 1985), reindeer (*Rangifer tarandus*), Chamois (*Rupicapra rupicapra*), thar (*Hemitragus emlaicus*) and steenbok (*Raphicerus campestris*) (Robinson and Balassu, 1981). The sources of these infections were thought to be orf of ovine or caprine origin, but no proof was presented.

Figure 1.1: Colour photographs of natural orf virus infections. (A) typical orf lesions on the lips and around the nostrils of an adult Saudi sheep (Nagdi breed); (B) nasty orf lesions involving lips and gum of a lamb (Nagdi breed).

A



B



Orf has not been observed in cattle that are mixed with infected sheep. Experimental infection of cows wasn't successful, but calves were susceptible to experimental infection on the muzzle (Robinson and Balassu, 1981).

1.3.5b Experimental infection of laboratory and some wild animals

Several investigators have studied orf infectivity in other animals. Guinea-pigs and mice were refractory to CPD virus (Blanc *et al.*, 1922; Aynaud, 1923; Jacotot, 1926; Glover, 1928; Greig, 1956; Darbyshire, 1961; Trueblood and Chow, 1963). However, successful infection of guinea-pigs was claimed by Selbie (1944), who used CPD virus passaged in rabbits. Reports of producing orf lesions experimentally on rabbits is controversial. Several workers were able to demonstrate orf infectivity in rabbits (Lanfranchi, 1925; Selbie, 1944; Wheeler and Cawley, 1955; Abdussalam, 1957b; Darbyshire, 1961). On the other hand, Aynaud, (1923); Glover, (1928); Howarth, (1929) and Greig, (1956) failed to do so. It is possible that the type and breed of the rabbit may play a role.

While the chorioallantoic membrane of chicken embryos has been used routinely for the propagation of most orthopoxviruses (Newsom and Cross, 1934a; Greig, 1956; Darbyshire, 1961), only Sawhney and Spasova (1973), have claimed multiplication of orf virus on chorioallantoic membrane. Chickens, pigs, dogs, cats, pigeons and frogs were refractory to CPD infection (Aynaud, 1923). Monkeys were found to contract experimental CPD infection (Bennet *et al.*, 1944). Experimental infection was demonstrated successfully by Lance *et al.*, (1983) on mule deer fawns (*Odocoileus hemionus*), wapiti calves (*Cervus elaphus nelsoni*), pronghorn fawns (*Antilocapra americana*), and white-tailed deer fawns (*Odocoileus virginianus*) using orf infective material obtained from rocky mountain sheep. It would appear that sheep and goats are the most suitable and susceptible species for experimental orf infection.

1.3.6 HUMAN ORF

Orf is a zoonotic disease. The first report of a case of human orf was confirmed by Newsom and Cross (1934a) who experimentally produced orf on the scarified thigh of a sheep with a sample collected from a man who had contracted the infection after treating sheep with sore mouth.

Human orf is an occupational disease, principally of farmers, abattoir workers, sheep shearers and veterinarians (Kewish, 1951; Hunter, 1964; Lyell and Miles, 1950; Nietzsche and Lomholt, 1980; Robinson and Balassu, 1981 and Huerter *et al.*, 1991), sheep ranchers (Erickson *et al.*, 1975) and retail butchers and meat workers (Robinson and Peterson, 1983).

The incubation period of the disease in humans is a bit longer than it is in sheep at between 3 and 7 days (Hunter, 1964). As in sheep, human orf lesions are usually self limiting with the course of the malady being between three and five weeks (Hodgson-Jones, 1951; Hunter, 1964).

The majority of human orf cases are reported to have resulted from direct contact with affected animals (Newsom and Cross, 1934a; Nagington and Whittle, 1961; Leavell *et al.*, 1968; Erickson *et al.*, 1975; Nietzsche and Lomholt, 1980; Huerter, Alvarez and Stinson, 1991). Orf, however, has also been reported to have occurred in people who had no direct contact with sheep, and it is believed that contaminated wool hooks, sheepyard rails and clothing can be sources of infection (Kewish, 1951; Leavell *et al.*, 1968; Robinson and Balassu, 1981; Huerter *et al.*, 1991). Man-to-man infection has been reported (Lang, 1961). Re-occurrence of human orf was described by Gourreau *et al.*, (1986), who diagnosed orf in a farmer on the ulnar aspect of her hands and wrist, which resolved in 21 days. Eight months later, the woman developed orf lesions on the median aspect of her right wrist.

The effect of orf in humans has been reported to vary. Lesions are commonly solitary and have been found on different parts of the body: fingers, arms, hands,

temple, chin and face (Nagington and Whittle, 1961; Leavell *et al.*, 1968; O'Neill *et al.*, 1990; Huerter *et al.*, 1991). Multiple lesions have also been described. Kewish (1951) reported lesions on the arms, face and trunk of an affected woman accompanied by an acute febrile reaction. Erickson *et al.*, (1975) noticed generalised contagious ecthyma in a sheep rancher, in which lesions extended to involve the face, arms, shoulder, axilla, lower abdomen, legs and feet. Severe and complicated cases have also been described by Royer *et al.*, (1970) and Huerter *et al.*, (1991). Hands, feet, legs, eyes, scrotal and perianal sites were affected, and lymphadenopathy, lymphadenitis, lymphangitis and permanent blindness were also reported. Very severe orf lesions have been observed in a man who had received severe electrical burns whilst working on overhead power lines and fallen into a sheep yard. The lesions were severe, widespread and coincided with the skin burns (A.A. Mercer, Personal communication).

Orf in humans is reported to have 6 different stages. They are: The Maculopapular stage, which lasts 1-7 days, and consists of erythema, spots and slight elevation of the skin. The Target stage, lasts 7 to 14 days when the lesion has a red centre surrounded by a white ring. The Acute stage, 14 to 21 days, when there is weeping at the surface of the lesion. The Regenerative stage, 21 to 28 days, when the lesion dries. The Papillomatous stage, 28 to 35 days, when papilloma develop on the surface of the lesion, and finally the Regressive stage, when there is reduction of lesion size, the crust drops off and healing occurs (Leavell *et al.*, 1968).

Prevention of orf infection in people who are regularly in contact with animals such as farmers and abattoir workers, will mainly depend on the reduction and control of orf in sheep and goats.

1.4 THE AGENT

1.4.1 STRUCTURE AND MORPHOLOGY

Orf virus is a member of the poxviridae, genus parapox, along with milker's node (MN) (pseudocowpox), bovine papular stomatitis (BPS), contagious ecthyma of

camels, parapoxvirus of red deer, and the tentative seal and squirrel parapoxviruses (Nagington *et al.*, 1964; Wilson *et al.*, 1972; Menna *et al.*, 1979; Hadlow *et al.*, 1980; Setien *et al.*, 1980; Sands *et al.*, 1984; Rafii and Burger, 1985; Munz *et al.*, 1986; Hicks and Worthy, 1987; Horner *et al.*, 1987; Osterhaus *et al.*, 1990; Binns and Smith, 1992; Osterhaus *et al.*, 1994; Nettleton *et al.*, 1995).

Orf virus particles measure approximately 260 x 160nm and are more ovoid than the characteristically brick-shaped orthopoxviruses (Abdussalam and Cosslett, 1957; Nagington and Horne, 1962; Harkness *et al.*, 1977).

Two predominant interchangeable forms of orf particles have been demonstrated by phosphotungstate stain: Mulberry forms (M) which have a ball of wool appearance, attributed to the criss-crossing of tubular protein threads on the surface of the virion and clear forms (C) which are phosphotungstate permeable (Robinson and Balassu, 1981; Robinson *et al.*, 1982; Binns and Smith, 1992). The two forms have also been detected in seal parapox virus preparations (Simpson *et al.*, 1994).

Ultrathin sections of negatively stained orf virus particles were described to have an inner S shaped tubular structure surrounded by a contrasted matrix, surrounded by a lipoprotein envelope (Nagington and Horne, 1962; Robinson and Balassu, 1981).

Relatively little is known about the genome of orf virus and the other parapox viruses in contrast to the vaccinia (orthopoxvirus) genome which has been completely sequenced. Orf virions contain double stranded, linear DNA with 63% G+C content. Milker's node and BPS viruses were found to share the same G+C content (Wittek *et al.*, 1979; Thomas *et al.*, 1980; Robinson and Balassu, 1981; Robinson *et al.*, 1982; Mercer *et al.*, 1987; Fleming *et al.*, 1993 and Lyttle *et al.*, 1994).

The size of genome of some parapoxviruses assessed by summation of the molecular weight of their fragments was found to be 88.8×10^6 (140 Kb) for orf virus, 87×10^6 for MNV and 86×10^6 for BPSV (Menna *et al.*, 1979; Thomas *et al.*, 1980; Robinson

and Balassu, 1981; Robinson *et al.*, 1982; Rafii and Burger, 1985; Binns and Smith, 1992). Vaccinia virus DNA has a 122×10^6 molecular weight (187 Kb) which is double stranded and linear with a low G+C content (36%) compared to parapoxviruses (Mercer *et al.*, 1987; Rodriguez *et al.*, 1987; Johnson *et al.*, 1993).

Cross-linked ends in Orf, MN and BPS viruses have been demonstrated by DNA-DNA hybridisation as well as for vaccinia DNA, suggesting that cross-linked ends are common to all members of the poxviridae (Wittek *et al.*, 1978; Menna *et al.*, 1979 and Gassmann *et al.*, 1985).

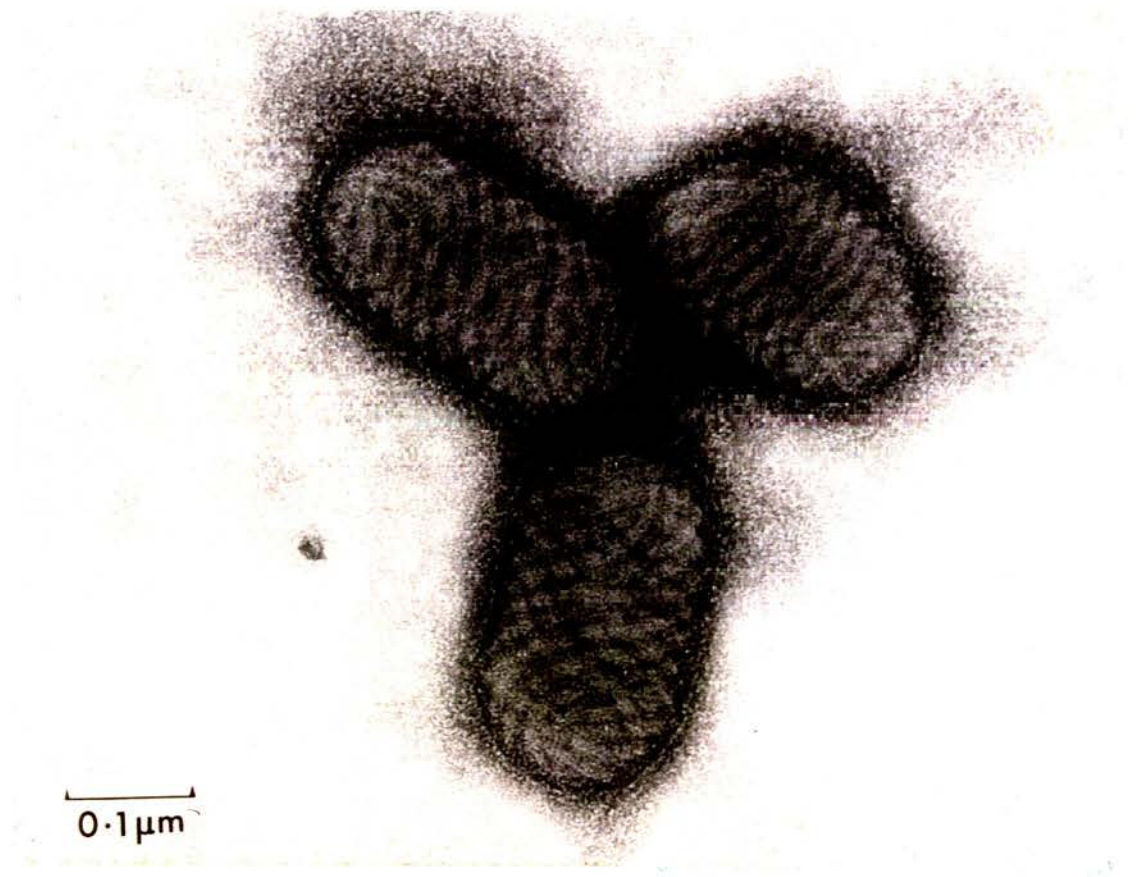
1.4.1a Restriction endonuclease profiles

Genetic heterogeneity among parapoxviruses has been reported by several workers (Wittek *et al.*, 1980b; Robinson *et al.*, 1987; Mazur *et al.*, 1991). Furthermore heterogeneity within strains of the same virus group is observed (Robinson *et al.*, 1982; Binns and Smith, 1992).

Isolates of Orf, BPS and MN viruses were studied by Wittek *et al.*, (1980b) and Gassmann *et al.* (1985). They reported different restriction profiles between the three virus groups and the existence of heterogeneity even between strains belonging to the same group. In contrast, orthopoxviruses have been shown to have more similarity to each other (Mackett and Archard, 1979; Gassman *et al.*, 1985). Recently, restriction endonucleases have been used successfully to differentiate the parapoxvirus of red deer from BPS virus and MN virus and orf virus (Robinson and Mercer, 1995).

Variation in the restriction profile can be attributed to minor changes in the DNA sequence resulting from deletion, insertion or inversions. In one variant of WR vaccinia virus, for example, one variant was believed to have lost DNA originally present in the parent virus (Mackett and Archard, 1979; Panicali *et al.*, 1981; Rafii and Burger, 1985).

Figure 1.2: Electron micrograph of orf virus, showing the typical crossing tubules of the virus envelope (Courtesy of E W Gray).



1.4.1b Hybridization

DNA-DNA hybridisation of parapoxviruses shows conservation in the central region of the genomes. Similar conservation of central genes has been seen in orthopoxviruses. There is less homology in terminal regions which tend to be more species specific (Mackett and Archard, 1979; Menna *et al.*, 1979; Gassmann *et al.*, 1985; Rafii and Burger, 1985; Fenner, 1990; Binns and Smith, 1992).

Robinson and Mercer (1995) have used parapoxvirus of red deer (DPV) as a probe to study its homology with orf, MN and BPS viruses. The DPV was found to have terminal region heterogeneity with all other three viruses by the DNA hybridisation (Figure 1.4). Therefore, DPV was reported to be the fourth parapoxvirus.

Mazur *et al.*, (1991) used the Thymidine Kinase (TK) gene of vaccinia virus as a probe to detect the TK gene of orf virus DNA in Southern blots to illustrate a similarity among members of the poxviridae. Interestingly, Orf virus TK gene was reported to locate on a 5.6 Kb HindIII digest fragment nearly similar to that of vaccinia which locates on a 5 Kb HindIII fragment (Boyle and Coupar, 1986; Groebel *et al.*, 1990; Mazur *et al.*, 1991).

The orf virus genome is described in this text chronologically as it first appeared in the literature. Lack of detailed information about orf virus isolates and other parapoxviruses at the genomic level makes it easy to review them. So far, only a few reports have been published on the NZ2 strain of orf virus. Nevertheless, from the sequencing of selected regions, it has emerged that, there is considerable conservation of structure and arrangement of genes between orf (OV) and vaccinia virus (VAC).

Orf virus encodes a pseudoprotease (orf-pp) near the 5' end of the DNA. The gene is known to occur in vaccinia virus but has also been reported in retroviruses namely, simian retrovirus (SRV), simian Mason Pfizer virus (SMPV), human endogenous retrovirus (HERV), squirrel monkey retrovirus (SMRV), maedi-visna virus of sheep

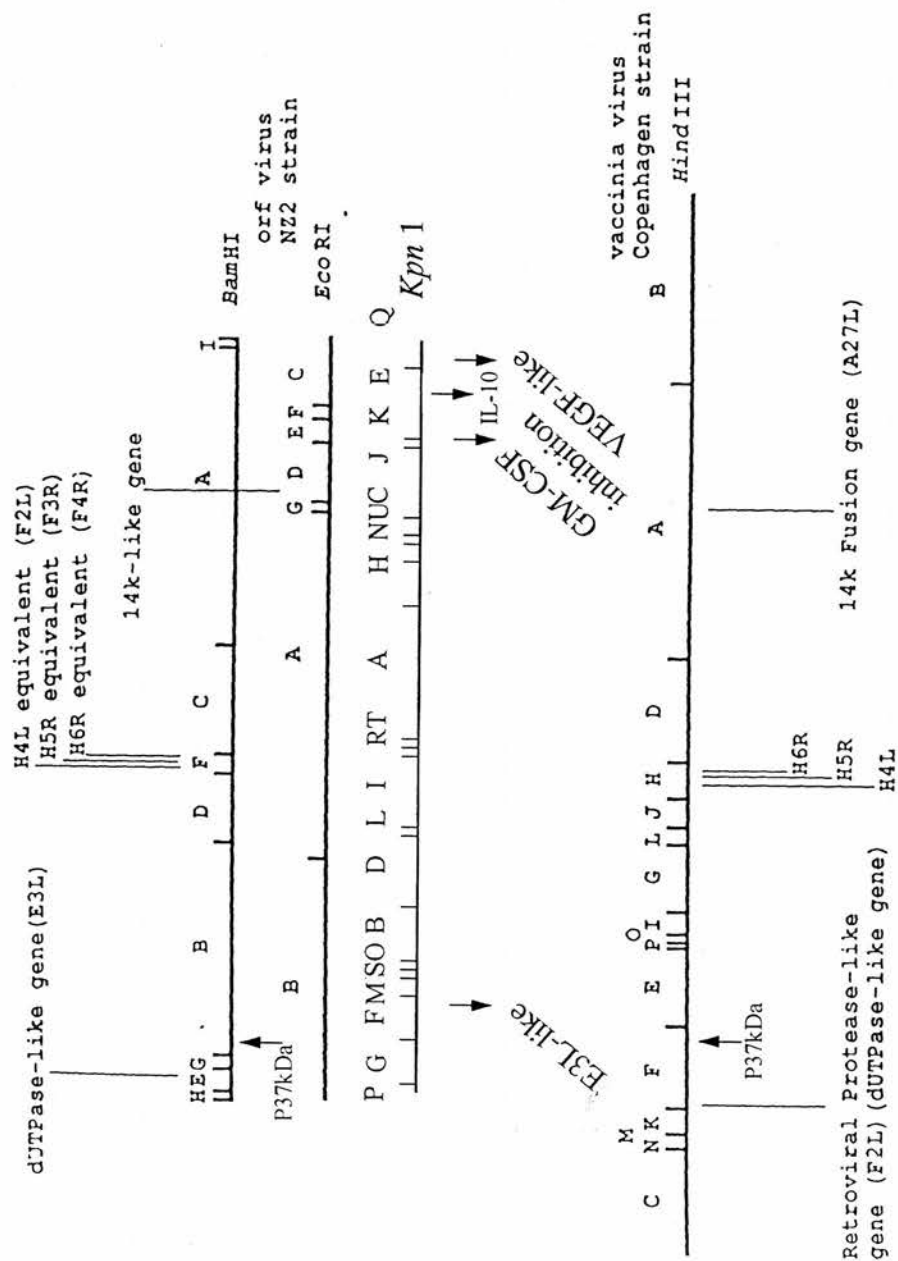
(M-VV), equine infectious anaemia virus (EIAV) and mouse mammary tumour virus (MMTV) (McClure *et al.*, 1988; Slabaugh and Roseman, 1989). Up to 28.3% homology was noted between orf-pp and pseudoproteases of each of the retroviruses mentioned above. Moreover, the residues of orf-pp and the residues of retrovirus-pps are reported to be conserved. Four strings of conserved residues located at position 31, 86, 113 and 141 were common among most of the retroviruses (Mercer *et al.*, 1989). The role of pseudoproteases in retroviruses and poxviruses has yet to be determined.

Three open reading frames in the inverted terminal repetition (ITR) were obtained in the right-hand region of the orf virus genome in two BamHI fragments (BamHI E and BamHI I). In a total sequence of 4425 base pairs, up to 8 open reading frames (ORFs) were observed, three of which were found within the ITR. The three ORFs of the ITR are believed to be transcribed early (Fraser *et al.*, 1990).

The role of the ITRs is still unclear. It has been suggested that they involve and enhance replication of viral particles, but another concept is that they are functionless and may have resulted from uneven recombination events and replication errors (Wittek *et al.*, 1978 and Fraser *et al.*, 1990). Since they are known to be transcribed, they are probably involved in viral replication in one way or another.

Presence of ITRs in the genome of orf, papular stomatitis, vaccinia and fowl pox viruses indicate that ITRs may be a characteristic feature of the poxviridae (Gassmann *et al.*, 1985; Wittek *et al.*, 1978; Fraser *et al.*, 1990; Binns and Smith, 1992).

Figure 1.3: Orientation of the orf virus NZ2 genome map with that of the Copenhagen strain of vaccinia virus. EcoR1, BamH1 and Kpn1 maps of the orf virus are shown in alignment with the HindIII map of vaccinia virus. Orf virus genes identified so far are shown in relation to their vaccinia equivalent (Fleming *et al.*, 1993; Sullivan *et al.*, 1994; Haig *et al.*, 1997).



A 30 Kb nucleotide sequence from the right hand end of orf NZ2 in the EcoRI D fragment was sequenced by Naase *et al.*, (1991). They revealed that the sequence encodes a 10kDa peptide and they described 31% amino acid similarity between the 10kDa peptide and the 14kDa fusion protein of vaccinia virus. However, in the alignment of the two viral proteins there were noticeable differences: vaccinia virus amino acid residues 28 to 51 were absent from the orf virus 10kDa protein which was 89 rather than 210 residues long. Also the potential glycosylation site of amino acids NVT at positions 60 to 62 in vaccinia virus was absent in orf virus (Naase *et al.*, 1991).

Shortly afterwards the 3.3 Kb BamH1F fragment located in the centre of the orf virus NZ2 was studied. Three major open reading frames (ORFs) were found from sequencing that fragment, designated as F2L, F3R and F4R encoding proteins similar to that of vaccinia H4L (RNA polymerase-associated protein RAD94), H5R (35kDa virion envelope antigen) and H6R (topoisomerase) (Fleming *et al.*, 1993; Kiemperer *et al.*, 1995). The OV ORFs and VV ORFs were found to be located in a nearly identical manner, in spite of them belonging to different genera and having variable G+C contents. This further confirms conservation of genes at the centre of poxviruses even those in different genera.

Another gene has been identified 10Kb from the left end of the orf virus NZ2 genome in BamH1 fragment B, encoding a protein of molecular weight 42kDa. It has been found that this 42kDa orf virus protein has an amino acid sequence similarity around 42% to the 37kDa protein of a VV major envelope antigen, 42.8% to a 43kDa protein of molluscum contagiosum virus and up to 38.3% with a 43kDa protein of fowlpox virus, thus illustrating the existence of similarity of this protein in poxviruses (Sullivan *et al.*, 1994). Recombinant 42kDa protein induced a good antibody response in sheep and is the first immunogenic parapoxvirus protein whose gene has been identified.

A gene encoding a polypeptide that has homology to mammalian vascular endothelial growth factor (VEGFs) has been demonstrated in the orf virus genome. Two New Zealand orf virus strains NZ2 and NZ7 have been examined and it has been shown in both that the gene is transcribed early in infection and is located in the right end of the genome before the inverted terminal repeat. The two VEGF genes were reported to encode variant polypeptides of molecular weight 14.7kDa in NZ2 and 16kDa in NZ7. They showed variable identity to the mammalian VEGF up to 27% by NZ2 and up to 23% by NZ7, and were found to be 41% identical to each other (Lyttle *et al.*, 1994). Presence of distinguishable VEGF genes in two orf viruses is thought to have resulted from acquirement of the gene from the mammalian host and subsequent genetic drift. Comparative studies, involving strains from different geographical parts of the world, to determine whether all orf virus strains have VEGF genes are required. The way by which VEGF may be useful to the virus is not obvious. Nonetheless, it is believed that it increases the flow of metabolites essential for the virus, by indirectly increasing blood supply to the skin (Lyttle *et al.*, 1994; Yirrel *et al.*, 1994).

A gene coding for the homologue of interleukin 10 (IL-10) has been described in orf virus (Fleming *et al.*, 1997). The gene was localised in the right end of kpn1 fragment E. The length of the polypeptide encoded by the gene was 186aa, with a molecular weight of 21.745kDa.

The IL-10 sequence of orf virus was shown to have nearly 80% amino-acid identity with the sheep IL-10; 75% with that of cattle; 67% with human IL-10 and 64% with mouse IL-10. The role of the orf virus IL-10 is unknown but mammalian IL-10 is known to be a multifunctional cytokine with suppressive effects on inflammation, antiviral response and T-helper type 1 (Th1) function (Mossmann, 1994; Fleming *et al.*, 1997).

The orf virus IL-10 is believed to be ovine IL-10 captured by orf virus in recent orf virus evolution (Fleming *et al.*, 1997).

1.4.1c Polypeptides

From purified orf virions up to 35 polypeptides ranging in size from 10 to 130kDa have been demonstrated using polyacrylamide gel electrophoresis. Thirteen of the 35 polypeptides were found in a detergent-soluble fraction of the virus envelope (Balassu and Robinson, 1987). Zuo *et al.*, (1988) found 28 polypeptides in purified orf virus preparations. From the viral 'capsule' 18 viral subunit proteins were visualised. The molecular weights of the major ones were 4.5, 33.1, 38.0 and 40.7kDa. Furthermore, each subunit was shown to produce antibodies against their native proteins in whole virus and produced cellular immunity after being injected into the skin of lambs. In another parapoxvirus, MN, 40 polypeptides were seen between 10 and 200kDa (Thomas *et al.*, 1980; Binns and Smith, 1992). In vaccinia virus (orthopoxvirus) up to 111 polypeptides have been resolved in two-dimensional (2-D) gel electrophoresis (Essani and Dales, 1979). It has been estimated up to 263 potential proteins are encoded by vaccinia virus (Johnson *et al.*, 1993).

The polypeptide profiles of orf virus strains are generally similar to each other. However, based on the appearance of polypeptides ranging from 37 to 44kDa Buddle *et al.*, (1984) classified 11 orf virus isolates into 4 groups. The same observation has previously been described for orthopoxviruses that can be differentiated from each other according to the presence of polypeptides between 30,000 to 40,000 daltons (Ikuta *et al.*, 1978; Arita and Tagaya, 1980). Contrary to these reports five New Zealand orf virus strains were examined and no significant difference could be demonstrated between them based on the major basic subunit polypeptide of 38kDa molecular weight (Balassu and Robinson, 1987). This 38kDa polypeptide is believed to be the basic subunit structure of the surface tubules of orf virus. Balassu and Robinson (1987) are of the opinion that the 38kDa polypeptide of the NZ isolates is apparently similar to the 37 to 44kDa polypeptide of North American viruses reported by Buddle *et al.*, (1984).

In an attempt to examine whether passaging the orf virus in tissue culture has an effect on the proteins of the virus, two different isolates were tested in

polyacrylamide gel electrophoresis at passages 8 and 32. There was no significant difference between the two isolates at the two passage levels (Balassu and Robinson, 1987).

Thomas *et al.*, (1980) were able to demonstrate using polyacrylamide gel electrophoresis and electron microscopy that a 42,000 to 45,000 dalton protein is a major component of the surface tubule structure (Thread like) of MN virus. For orf virus a 40kDa protein has been shown to be the major component of the surface tubule (McKeever *et al.*, 1987).

It has been found using early immune sera from lambs that one of the first viral polypeptides recognised has a molecular weight of 40kDa by western blotting (McKeever *et al.*, 1987). A similar study using a monoclonal antibody, which detected by blotting a 40 to 43kDa polypeptide of orf virus also identified this as an early immunodominant protein; the same mab detected a 45 to 48kDa polypeptide of MN and BPS viruses (Lard *et al.*, 1991). These results confirm the early report of Thomas *et al.*, (1980) and show the existence of common proteins between OV, MNV and BPSV.

Recently, a comparative western blot analysis showed that capripoxvirus (cpv) hyperimmune serum recognised 67, 32, 26, 19 and 17kDa proteins from purified cpv, and also recognised 67, 42, 32 and 18kDa proteins in purified orf virus. Orf virus hyperimmune serum identified 5 homologous polypeptides, and also cross-reacted with 26, 42 and 67kDa polypeptides of cpv, but not the 32kDa protein (Chand *et al.*, 1994).

1.4.2 CLASSIFICATION OF ORF AND OTHER PARAPOXVIRUSES

The name poxviridae originated from the plural of the old English "POCC" which means a vesicular skin lesion (Dinter and Morein, 1990).

Parapoxviruses are members of the family poxviridae, subfamily chordopoxvirinae (vertebrate viruses) (Figure 1.4). They have a distinctive appearance under the electron microscope (Fenner *et al.*, 1987; Francki *et al.*, 1992; Wyler, 1994).

In 1956 parapoxviruses were classified and included two viruses, Orf and MN. A few years later the International Committee on the Taxonomy of Viruses (ICTV) chose orf virus as the type species of the parapoxviruses which also included MN and BPS viruses (Binns and Smith, 1992).

Orf, MN and BPS viruses are classified based on the species affected, the pathological picture of the malady, and are distinguished from other poxviruses by their electron microscopic appearance (Binns and Smith, 1992).

The latest review by the poxvirus study group of the ICTV include within the parapox genera: Orf, MN and BPS viruses. Probable new members identified are: contagious ecthymas of camels, seal parapoxvirus, parapoxvirus of red deer and squirrel parapoxvirus (Figure 1.4). It is not clear whether to consider contagious ecthyma of chamois as a new member or whether it is orf virus, since clinical orf has been reported in chamois (Robinson and Balassu, 1981; Fenner *et al.*, 1987; Binns and Smith, 1992; Franki *et al.*, 1992).

Gassmann *et al.*, (1985) reported that the differentiation of Orf, MN and BPS virus is justified according to the terminal analysis of the genomes by cross-hybridisation. Robinson and Mercer (1995) confirmed these finding and have additionally proved that the parapox of red deer is a fourth distinct parapoxvirus (Figure 1.5). However, their finding has yet to be supported by the ICTV to be fully accepted.

Figure 1.4: The Poxvirus Group

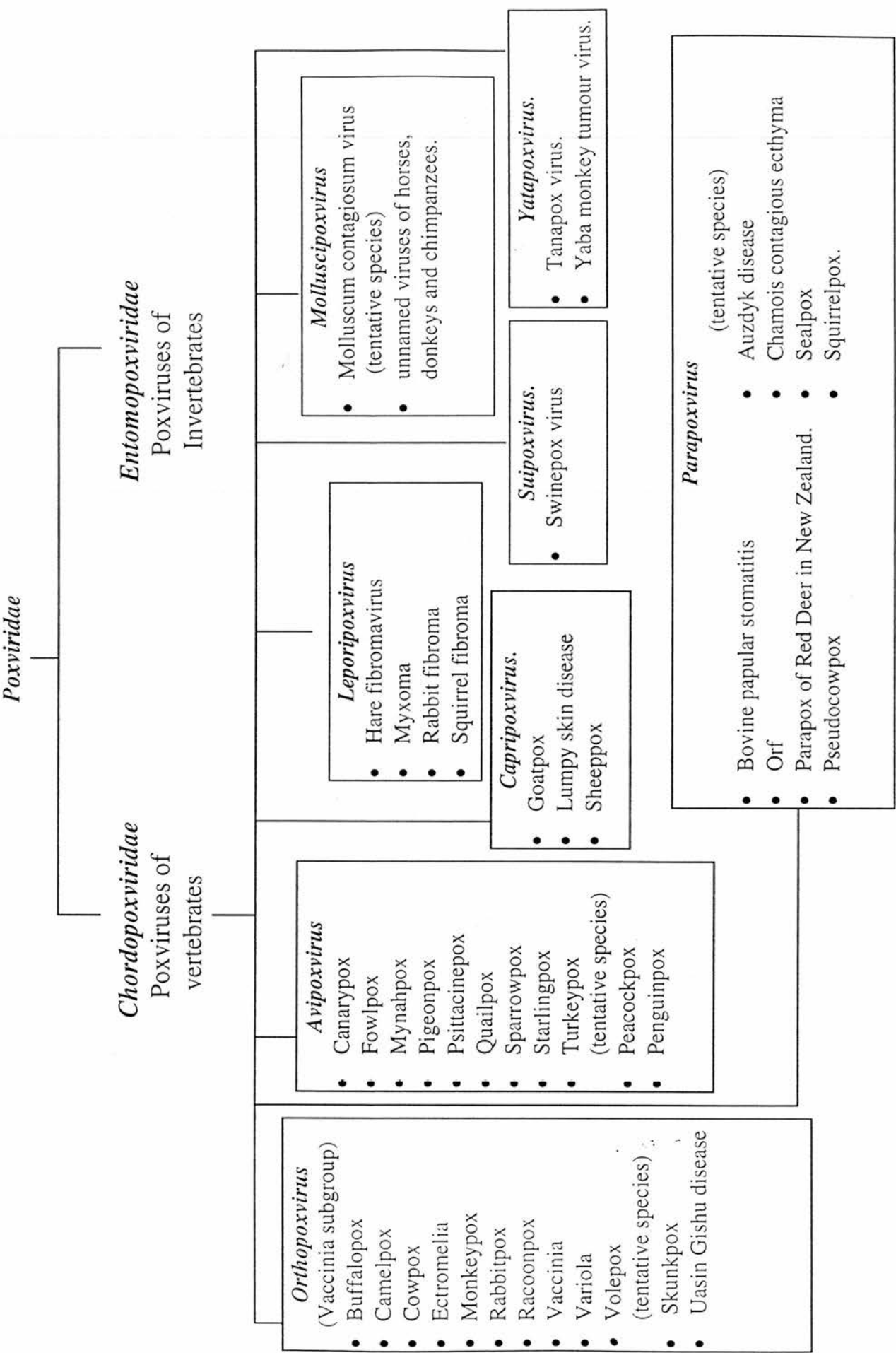
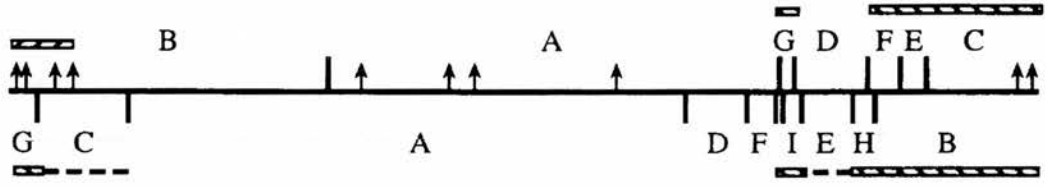
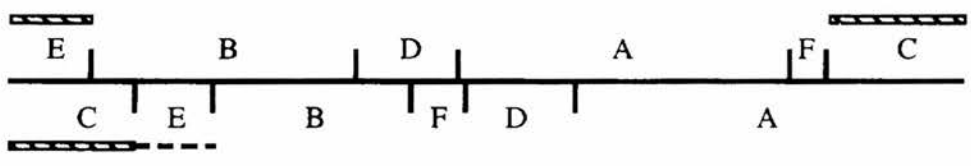


Figure 1.5: Site of parapoxvirus genomes showing non-homology with DPV DNA on restriction endonuclease cleavage site maps of OV, BPSV and pseudocowpoxvirus (Milker's node). Cleavage sites for EcoRI are marked by vertical bars extending above the maps and HindIII sites by bars extending below. Restriction fragments of each genome which did not hybridise with a DPV DNA probe are indicated by cross-hatched bars. Fragments which showed only very weak hybridisation are indicated by dashed lines (Robinson and Mercer, 1995).

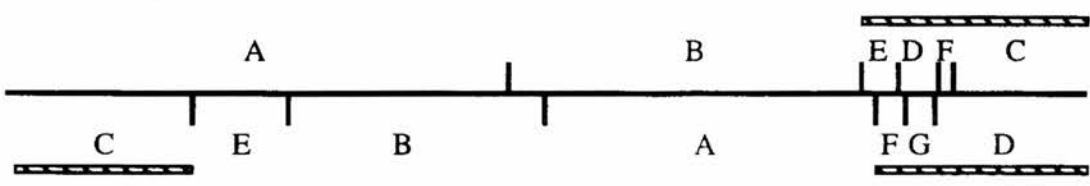
Orf Virus



Bovine Papular Stomatitis Virus



Pseudocowpox Virus



20 kb

1.4.3 STRAIN VARIATION OF ORF AND OTHER PARAPOXVIRUSES AND THEIR RELATION WITH CAPRIPOXVIRUS AND ORTHOPOXVIRUS

There is a traditional view that orf virus strains from different parts of the world are antigenically similar. This view has arisen from early work comparing several orf viruses serologically or by cross-protection in sheep and goats. English and Australian strains were reported to be similar and immunologically indistinguishable by cross-protection (Seddon and McGrath, 1931). One year later Glover (1932), stated that orf virus strains from sheep in England, California and France were identical and also English and Tanganyika goat isolates were similar. Later Horgan and Haseeb (1947) studied the cross-immunological relationship of three orf virus isolates from Cyprus and two from England. They didn't detect significant differences between Cyprus 1, 2 and the English strains, but Cyprus 3 was less similar. A study of strains from England, Bulgaria, Slovakia, Iran and Rumania, found that the Rumanian and Slovakian strains were identical and the English and Bulgarian strains were similar. Moreover, a similarity between the Iranian and the English strains was noted, but the Iranian and Bulgarian isolates were distinguishable, indicating polyvalence of orf strains (Sawhney, 1966). When Precausta and Stellmann (1973) tested five different orf viruses by serum neutralisation, the viruses showed a close relationship to each other. Reid (1991) has stated in the light of cross-protection results that viruses from the United Kingdom, USA, Australia and France are similar.

Occurrence of the disease in different forms from mild to very aggressive based on the lesion and mortality rates demonstrates variable pathogenicity among orf virus strains (Aynaud, 1923; Glover, 1928; Bragazzi, 1959; Darbyshire, 1961; Robinson and Balassu, 1981; Mazur and Machado, 1989). The malignant form of orf described by Darbyshire (1961), affected sheep in Kent, with lesions extending to involve the gastrointestinal tract. The same serious disease has also been reported recently in Australia, (Hawkins *et al.*, 1991).

While the serological and cross-protection data generally fail to differentiate orf viruses, genetic heterogeneity has been clearly demonstrated as described in section (1.4.1a) (Wittek *et al.*, 1980b; Robinson *et al.*, 1982).

Bovine papular stomatitis (BPS) virus isolates from Dorset, Scotland and Germany were compared by Nagington *et al.*, (1967), for their growth in five cell types, including primary human amnion (Am), bovine testes (BT), sheep testes (ST), secondary rhesus monkey kidney (Mk) and Hela cells. They found that all five Dorset strains grew in Am and ST cells, one of the three Scottish strains grew on Am and St cells, whereas, three of the five German strains grew in ST and Mk cells, indicating some variation in their growth in tissue culture.

No serum-neutralisation or cross-protection studies have been made to determine variation among strains of BPS or Milker's node strains (Reid, Personal communication). Restriction endonuclease studies, however, have demonstrated clear differences among and between BPS and MN isolates (Wittek *et al.*, 1980b and Gassmann *et al.*, 1985).

Vaccinia virus is the type species of the genus Orthopoxvirus. All the viruses in the genus orthopoxvirus are inter-related, based on cross-protection results (Muller *et al.*, 1977; Mayr and Czerny, 1990).

Orf (parapoxvirus) and vaccinia (orthopoxvirus) do not cross-protect either *in vivo* or *in vitro* (Robinson and Mercer, 1988; Mercer *et al.*, 1994; Wyler, 1994).

1.4.4 PHYSICO-CHEMICAL PROPERTIES

Poxviridae are resistant to adverse conditions and orf virus is no exception. Several authors have reported persistence of the virus in scabs for long periods. In dry scabs at cool temperatures the virus can remain infective for a period up to 23 years (Glover, 1928; Hart *et al.*, 1949; Livingston and Hardy, 1960; Buxton and Fraser, 1977; McKeever and Reid, 1986; Reid, 1991; Wyler, 1994). In moist scabs, however, the infectivity falls quickly (Reid, 1991). McKeever and Reid (1986) found

that rainfall had dramatically reduced orf virion infectivity, attributed to hydration of the scab material.

Exposure of the scabs to direct sunlight is noted to cause reduction and eventually remove infectivity of the virus (Boughton and Hardy, 1935; Robinson and Balassu, 1981). However, McKeever and Reid, (1986) are of the opinion that under natural conditions UV radiation has little effect on the virus, due to the protection of the virus by the scab. Experimental exposure of the virus in suspension for 30 minutes to ultra-violet light (UV) resulted in loss in virus titre of tissue culture grown virus between 1.8 and 2.6 Log₁₀ TCID₅₀ (Sawhney, 1972).

Drying and powdering the scabs over sulphuric acid and storage in a sealed container in a cold room was found to maintain virus potency for up to 33 months (Glover, 1928; Boughton and Hardy, 1935; Livingston and Hardy, 1960).

Orf virus is resistant to heat and remains infective at 55°C for 30 minutes. Inactivation has been reported to occur at 60°C for 30 minutes, 56°C for 1 hour and 100°C for 2 minutes (Boughton and Hardy, 1935; Greig, 1956; Plowright *et al.*, 1959; Sawhney, 1972; Buxton and Fraser, 1977; Sinha and Soman, 1988; Saddour, 1989).

Evidence for the effect of solvents on orf virus is conflicting. Ether was found to have no effect on the infectivity of orf virus (Glover, 1928; Trueblood and Chow, 1963; Saddour, 1989; Reid, 1991). Contrary to this, Seddon and Belschner (1929), have described orf virus inactivation by ether. Chloroform can be used successfully to inactivate orf virus (Seddon and Belschner, 1929; Saddour, 1989; Reid, 1991).

1.5 IMMUNITY

1.5.1 ACTIVE IMMUNITY

Monitoring the development of active immunity in sheep and goats to orf virus infection was reported some time ago. In Italy, sheep recovered from natural or experimental orf virus infection were immune and protected for five to eight months

(Lanfranchi, 1925). In the UK, Glover (1928) found that immunity started 9 to 15 days post-infection, and lasted for eight months. In Australia, Seddon and Belschner (1929) reported that following natural or experimental infection sheep and goats possess a solid immunity to re-infection. They also demonstrated that skin immunity involved the whole body and was not localised to sites where primary lesions had occurred. However, they did not report how long immunity lasted. Other investigators have confirmed generalised skin immunity and claimed that immunity could extend from 7 to 24 months (Howarth, 1929; Schmidt and Hardy, 1932; Boughton and Hardy, 1935; Hart *et al.*, 1949; Robinson and Petersen, 1983).

In order to control and attempt to prevent the occurrence of the disease, live vaccination has long been practised. In 1923, Aynaud vaccinated sheep using a 1% suspension of dried scab in 50% glycerine in saline, and reported good protection against natural infection. Following Aynaud's recipe but with a reduction of the glycerine in normal saline to 25% Glover (1928) vaccinated sheep. He claimed that his vaccine was safe, induced a localised lesion and a solid protection. Another successful trial with the same type of vaccine was conducted by Hart *et al.*, (1949), who found that 0.01g of dried powdered scab in 10ml of the buffer was quite enough to vaccinate 500 lambs. In a massive study Boughton and Hardy (1935) used the same vaccine as Aynaud. They vaccinated around eight thousand lambs and reported good protection in general, although, the degree of protection varied to some extent between lambs.

Although they didn't mention the method by which their vaccine was prepared Kerry and Power (1971) were successful in demonstrating the safety of vaccination of one to two day old lambs. Nonetheless, 1% of the 2000 lambs they studied developed orf lesions around the mouth and nostrils, which they believed to be due to a mistake in the administration of the vaccine. Buddle and Pulford (1984) also successfully described vaccination of newly born lambs.

Vaccination of badly affected animals was noted to reduce the course of the disease (Boughton and Hardy, 1935; Robinson and Balassu, 1981). In Saudi Arabia under

field conditions, autogenous vaccination of infected animals has been practised. Scabs collected from the lips of sheep are applied directly on to a scarified region of the thigh of the same sheep. This has been reported to reduce the severity of the facial orf and aid recovery (Gameel, A, Personal communication).

For control of orf virus infection the recommended sites of vaccination are the medial aspect of the thigh and for pregnant ewes behind the axilla. Vaccination should be applied to pregnant ewes at least two months before lambing to ensure lambs are not exposed to vaccine virus neonatally (Aynaud, 1923; Glover, 1928; Boughton and Hardy, 1935; Hart *et al.*, 1949; Kerry and Powell, 1971 and Kovalev *et al.*, 1971).

It is been stated that the duration of the immunity in different parts of the animal varies from region to region. Lips and feet were found to possess protective immunity which lasted longer than in the thigh, abdominal skin, genital sites and udder (Kovalev *et al.*, 1971; Robinson and Balassu, 1981).

Since it is believed that scab-derived vaccine increases the virus pool in the environment, tissue culture vaccine (attenuated vaccine) has been used by several workers in trials to vaccinate sheep and goats against orf. In a study made by Kovalev *et al.*, (1971), a vaccine prepared in lamb kidney cell culture, was found to be capable of inducing active immunity similar in potency to that produced by scab vaccine, as proved by protection against virulent virus in skin challenge. A similar type of vaccine has been described (Ramyer, 1973).

More than one tissue culture vaccine has been tested under field conditions. In Germany, lamb kidney cell culture vaccine was tested on five thousand animals and shown to be safe in new-born lambs and pregnant ewes (Zach, 1979). Another potential vaccine (D1701) passaged and attenuated in heterologous cell cultures, when examined on about twelve thousand sheep was also shown to be safe (Mayr *et al.*, 1981). Although not yet studied under field conditions Pye (1990) has described tissue culture adapted vaccine, that experimentally induced skin lesion and conferred

good protection against challenge. When tested on specific pathogen-free (SPF) lambs, this vaccine was protective for at least six months (Nettleton *et al.*, 1996).

Although evidence of heterogeneity between orf and bovine papular stomatitis viruses has been reported (Gassmann *et al.*, 1985), in a cross-protection study Buttner (1981), was able to immunise cattle against bovine papular stomatitis using orf tissue culture attenuated virus which conferred protection against experimental challenge.

Despite the success of vaccinating sheep and goats with orf tissue culture vaccine, live scab-derived vaccine is still the major commercial product which is widely used. This could be due to the fact it is cheaper in relation to tissue culture vaccine and is more readily available.

1.5.2 PASSIVE IMMUNITY

Whether or not colostral antibody from orf virus immune dams confers protective immunity to neonatal lambs and kids is still not resolved. Several authors have documented that while sheep recovered from natural infection gain a solid immunity, lambs born to the immune animals appeared to contract the disease, indicating no effective protection passed to them from their dams (Howarth, 1929; Seddon and Belschner, 1929; Glover, 1932-33; Boughton and Hardy, 1935; Kerry and Powell, 1971; Buddle and Pulford, 1984; Webster and Granoff, 1994).

Poulain *et al.*, (1972) and Jan *et al.*, (1978) have successfully found good levels of antibodies in the serum and colostrum of ewes immunised during pregnancy. Moreover, the titres of antibody correlated between the serum and colostrum of the ewes and between the colostrum and lambs serum. Verds *et al.*, (1989), have confirmed these earlier studies and obtained neutralising antibodies in both serum and colostrum. They have also claimed that lambs fed on colostrum from immune ewes are protected for 3 months.

Results of a comparative study between lambs receiving antibodies via colostrum and lambs being vaccinated on the first day of their life, showed that lambs that had colostrum were not protected against virulent virus despite the fact they possess high titres of antibody (Buddle and Pulford, 1984). Given the common occurrence of the disease in lambs and kids in the neonatal period it seems unlikely that colostral antibody is protective.

1.5.3 MECHANISMS OF IMMUNITY

1.5.3a Humoral responses

The early humoral response of sheep and goats to orf virus infection has been studied using several qualitative and quantitative serological techniques, namely complement fixation test (Rottgardt *et al.*, 1949; Abdussalam, 1958; Schmidt, 1967; Housawi *et al.*, 1992), neutralisation test (Abdussalam, 1958; Poulain *et al.*, 1972; Jan *et al.*, 1978), agar gel precipitation test (Abdussalam, 1958; Housawi *et al.*, 1992; Chand *et al.*, 1994), enzyme linked immunosorbent assay (ELISA) and western blotting (Koptopoulos *et al.*, 1982; McKeever *et al.*, 1987; Yirrell *et al.*, 1989; Housawi *et al.*, 1992; Housawi *et al.*, 1993). Antibody-mediated cytotoxicity has also been used (DeMartini *et al.*, 1978; Koptopoulos *et al.*, 1982). The humoral response of camels to a related parapox virus has recently been demonstrated using ELISA and western blot (Azwai *et al.*, 1995).

It has been stated that goats that have had goatpox infection and recovered are protected against goatpox and orf but not the other way round (Bennet *et al.*, 1944; Sharma and Bhata, 1959). Cross-reactivity has been confirmed serologically by several authors (Sharma and Dhanda, 1971; Dubey and Sawhney, 1979; Subba Rao and Malik, 1979). However, Renshaw and Dodd (1978), found no cross-protection between orf and goatpox both *in vivo* and *in vitro*.

From the cross-protection point of view between orf and goatpox, it will be interesting to know whether the reported protection is due mainly to humoral or cellular immunity.

Webster and Granoff (1994) indicate that recovery from orf is not clearly understood and the role of humoral immunity requires further studies.

In the light of clinical observation and serological evidence, many researchers believe that recovery and protection from orf is entirely due to cellular immunity (Robinson and Balassu, 1981; Binns and Smith, 1992; McKeever *et al.*, 1987), others suggest immunity is predominantly but not exclusively cell-mediated (Yirrell *et al.*, 1989; Webster and Granoff, 1994).

1.5.3b Cellular responses

An early cellular skin response to orf virus infection has been detected 24hr after experimental infection when a massive number of polymorphonuclear neutrophils were present. Infiltration of the large number of the neutrophils was attributed to their role in restricting penetration of the virus into the dermis, as well as producing an interferon like substance that has the ability to inhibit viral replication. A significant increase in the basophil cells was also observed, at 120hr post-infection, but the basophils role in orf infection has yet to be determined (Jenkinson *et al.*, 1990a,b).

A dramatic accumulation of class II dendritic cells underneath the site of infection has been commonly observed following orf virus infection (Jenkinson *et al.*, 1991; Lear *et al.*, 1996; Haig *et al.*, 1997).

MHC class II dendritic cells are normally found in the skin of the inner thigh in small numbers (Jenkinson *et al.*, 1991). Four subpopulations of MHC class II dendritic cells are reported to occur in normal thigh skin and they are grouped according to their anatomical location and expression of CD1 and Factor XIIIa antigens (Lear *et al.*, 1996). One dendritic cell type, Langerhans cells (LC) was described in the epidermis, and three Dermal dendritic cell (DC) types in the dermis, (Lear *et al.*, 1996).

Table 1.1: The dermal dendritic cell population of normal ovine skin (Lear *et al.*, 1996)

Cell Markers	MHC Class II	AchE	CD1	Factor XIIIa
Langerhans cells	+	+	+	-
Dermal DC [1]	+	-	+	-
Dermal DC [2]	+	-	-	+
Dermal DC [3]	+	-	-	-

Using limited markers (three Mabs) for ovine monocytes/macrophages, Lear *et al.*, (1996) found that none of the dendritic cells, either the resident ones or the ones that gathered following orf virus infection were expressing the macrophage associated antigens CD11a and CD11b, indicating they were not tissue macrophages. However, a wider range of markers may be required to identify the dendritic cells most important in responding to orf virus infection.

The precise function of MHC-Class II dendritic cells is not obvious (Table 1.1), nonetheless, it is presumed that they act as a barrier to prevent viral invasion into the dermis and eventually aid epidermal repair. Another suggestion is that they have antigen-presenting ability (Jenkinson *et al.*, 1991; Lear *et al.*, 1996; Haig *et al.*, 1997).

1.5.3b₁ In the skin

a) Naïve sheep

Lear *et al.*, (1996) used a group of SPF lambs to study the primary skin response to orf virus infection. For the first 48hrs MHC class II and Factor XIIIa dendritic cells were present underneath the infected epidermis, in the same numbers as in lambs with scarified skin alone. Increase of MHC-class II positive dendritic cells was recorded to peak at day 9 and maintained to day 30 post-infection, after which it declined to its pre-infection state. However, the peak of Factor XIIIa dendritic cells was detected at day 7 post-infection and dropped on day 19 to its pre-infection level.

Unlike the Factor XIIIa dendritic response to the primary infection described above Lear *et al.*, (1996) noticed the Factor XIIIa response after secondary orf infection appeared only transiently (for 48hrs) in a small number. It is presumed that the high number of Factor XIIIa DC that gathered in the primary orf infection probably has an immunological function in differentiating the primary from the secondary immune response. It would have been useful if Lear and her team had also included the picture of other mononuclear cells eg neutrophils, basophils in their investigation, in order that it could be compared with previously published work.

b) Previously infected sheep

In a very detailed experiment the appearance of T-cells and B-cells has been compared in scarified skin alone and scarified infected skin. A noticeable increase in the T and B cells was seen in scarified skin alone at 12hr post-scarification but 24hrs later the number of the cells returned to the pre-scarification state. That is not surprising and could be considered as a normal inflammatory response. On the other hand, in scarified infected skin examined at the same period a steady and remarkable increase in the number of T-cells was observed which peaked at 72hrs post-infection and then declined to the 12hrs level at 96hrs after infection. The number of B-cells was noted to fall and then increase to peak at 72hrs and gradually fall again to reach the minimum level at 120hrs. Populations of T-cell sub-types (T4, T8 and T19) were detected in the epidermis and pustule of the infected skin, while the B-cells were mainly seen in association with dermal blood vessels (Jenkinson *et al.*, 1992).

1.5.3b₂ Cannulated lymph

McKeever and Reid (1987) and Yirrell *et al.*, (1991) have studied the response of the skin of orf experimentally infected sheep by cannulating the efferent duct of the draining lymph node. They recorded production of lymphoblast cells carrying specific IgG against orf virus and lymphocytes expressing MHC class II antigen in lymph plasma.

Evidence of recruitment of CD8⁺ and CD4⁺ T-cells in the lymph compartment following orf virus infection has been detected by lymph cannulation (Haig *et al.*, 1992; Haig *et al.*, 1996d), indicating their involvement in the immunity against orf.

Orf virus was noticed to be able to replicate in re-infected sheep for several days, despite the presence of CD8⁺ T-cells. This would indicate that the cells are probably not immunologically sufficient to stop virus re-infection (Haig *et al.*, 1996d).

The CD4⁺ T-cells were found to predominate over the CD8⁺ cells in the lymphatic compartment and in the skin. Their role in speeding the recovery of the infection is yet to be determined (Haig *et al.*, 1997).

1.5.3b₃ The role of cytokines

Cytokines are soluble proteins produced by leukocytes and other cell types, which act as chemical communicators between cells (Callard and Gearing, 1994). Following skin re-infection with orf, four type of cytokines that may have an antiviral function have been detected - IFN- γ , IL-1 β , IL-8 and GM-CSF (Haig *et al.*, 1992; Haig *et al.*, 1996b; Haig *et al.*, 1996c; Haig *et al.*, 1997).

In an interesting study Haig *et al.*, (1992) compared the response of the IFN- γ in afferent and efferent lymph duct, after reinfection of the skin with orf virus. It was found that IFN- γ in the afferent duct appeared between 19-30hrs, while it took up to 4 days to be detected in the efferent duct. The late detection of IFN- γ in the efferent duct is attributed to the recruitment and activation of orf virus-specific cells within the lymph node first, thereafter the occurrence of an anti-orf virus immune response. Whereas, in the afferent lymph it was detected rapidly due to presence of activated memory T-cells in the skin.

Other potential anti-virus cytokines IL-1 β and IL-8 (interleukins) were found to occur rapidly (1st day) in the plasma of both afferent and efferent ducts, after re-infection

(Haig *et al.*, 1996a). It is believed that IL-1 β and IL-8 have chemokine activity, particularly neutrophil chemo-attraction.

It has recently been described that orf viruses possess mechanisms that interfere with the protective immune response (Haig *et al.*, 1996c). An anti-granulocyte-macrophage colony-stimulating factor (GM-CSF) binding protein is produced by orf virus. This activity is associated with a 60-70 KD protein detected in cell-free supernates (Haig *et al.*, 1997). The GM-CSF is presumed to be of great significance in organising and regulating phagocytic and antigen presenting function (Haig *et al.*, 1995).

The production of anti GM-CSF and other cytokines by orf virus is thought to at least temporarily inhibit protective immunity, so that the virus can replicate.

Orf virus also produces an interferon binding protein. Similar to the E3L gene of vaccinia virus the gene was found in the left hand end of the orf genome (KPN1-fragment F) (Mercer and Fleming, unpublished data). The E3L gene of vaccinia was noticed to inhibit interferon by releasing an interferon binding protein (Davies *et al.*, 1993). Evidence of such activity in orf virus has been demonstrated (Haig *et al.*, 1996c; Haig *et al.*, 1997).

Clearly a greater understanding of the cell-mediated immunity to orf virus is essential for the design of better immunisation strategies.

1.6 AIMS OF THE STUDY

The aims of the study were to produce and characterise monoclonal antibodies against orf virus; to use these mabs to identify orf virus proteins, to study the relationship among parapoxviruses and between parapoxviruses and other members of the poxviridae; and to develop antigen-capture ELISAs for the rapid diagnosis of orf virus infection.

CHAPTER 2
MATERIALS AND METHODS

2.1 VIRUSES

2.1.1 ORF-11

Isolated originally from a case of orf in a sheep in Scotland, it has been passaged 22 times on sheep thyroid cells, 5 times on FLM cells, 2 times on fetal lamb skin (FL-skin) and 2 times on FLM cells.

2.1.2 NZ-2

Isolated in BE testis cells from a case of orf in a sheep in New Zealand, (Robinson *et al.*, 1982). The virus was kindly supplied by Dr A A Mercer, University of Otago, Dunedin and grown in FL-skin for 3 passages and once on FLM.

2.1.3 D-1701

A German orf strain, propagated in lamb kidney cells 135 times, bovine lung (BL) cells 38 times, MA104 50 times and FLM twice (Czerny *et al.*, 1994b).

2.1.4 SCABBYMOUTH

An Australian orf virus isolate kindly provided by CSL laboratories (Pye, 1990). It was propagated twice on FLM at Moredun Research Institute.

2.1.5 BOVINE PAPULAR STOMATITIS (BPS-V660)

A German isolate of BPS kindly provided by Dr M Buttner, Federal Virus Laboratory, Tübingen, (Mayr *et al.*, 1981). It had been passaged on bovine embryonic lung 8 times and twice on FLM cells.

2.1.6 MILKER'S NODE (B074)

A German isolate, kindly provided by Dr M Buttner, Federal Virus Research Institute, Tübingen. Passaged three times on bovine embryonic lung and five times on FLM.

2.1.7 SEAL PARAPOX (BJ 820)

Isolated from a grey seal (*Halichoerus grypus*) in Scotland the virus had been passaged four times on FL-skin, once on foetal ovine cornea (FO-Cornea) and once on FLM (Nettleton *et al.*, 1995).

2.1.8 SQUIRREL PARAPOX

Isolated from a red squirrel (*sciurius vulgaris*) in England using red squirrel kidney cells, followed by 15 passages on FLM cells (Sands *et al.*, 1984).

2.1.9 MOREDUN REFERENCE SCAB (MRI)

Moredun reference scab (orf virus). Recovered from a Scottish field outbreak as scab material. Has been used periodically to experimentally infect sheep at Moredun from which further scabs have been collected. Number of sheep passages is not known.

2.1.10 W1866/2 - SCAB

Field scab, collected from an orf outbreak in a pedigree flock of Charolais sheep in England.

2.1.11 W2072 - SCAB

Field scab, collected from another orf outbreak in sheep in Britain.

2.1.12 SCABI-VAX

A live virus vaccine, licensed by Pitman-Moore Ltd, UK. However, the scab origin is thought to originally come from Australian sheep infected with orf.

2.1.13 OTHER SCABS RECOVERED FROM DIFFERENT ORF OUTBREAKS IN BRITAIN

- a - C093: scab material obtained from 8 month old Suffolk (lamb) suffering from orf disease.
- b - W1866/1: field scab, collected from orf infection in pedigree lambs in England
- c - C038: scab samples obtained from lambs in Little Hayfield, England, with history that orf lesions appeared on animals ten days post-vaccination.

d - W2076: scab material collected from orf outbreak in sheep in Middlesex, England.

W0766/1: scab material collect from orf infection in England.

2.2 CELLS

2.2.1 FOETAL LAMB MUSCLE (FLM)

A semi-continuous cell line. The cells were grown in 199 Medium supplemented with 10% foetal bovine serum (FBS), and were tested free of pestivirus. Cells were used between passage levels 3 and 23.

2.2.2 FOETAL LAMB SKIN (FL-SKIN)

Like the FLM cells, they are semi-continuous cells, grown in 199 Medium, plus 10% of FBS.

2.2.3 CV-1

African Green Monkey kidney cells line bought from European Collection of Animal Cell Cultures (ECACC), grown in DMEM medium, supplemented with 10% foetal bovine serum.

2.2.4 NSO

Myeloma cells of line NSO/1 derived from NS1/1 Ag 4.1 (Galfre and Milstein, 1981), grown and maintained at the Moredun Research Institute.

2.3 ANIMALS

2.3.1 BALB C MICE

The Balb/c mice have been in-bred at Moredun Research Institute for approximately 25 years. They were originally bought from a commercial breeder, Charles Rivers, UK Ltd. The Balb C mice were used for monoclonal antibody production.

2.3.2 LISTER RAT

The Lister rats descended from the PVG rat at Lister Institute (hence the name). Charles Rivers bought and bred at the Moredun Research Institute. The rats were used as thymus donors for mixed thymocyte medium.

2.3.3 WISTAR RAT

Wistar rat, the same as the Balb/c mice have been bred at Moredun for approximately 25 years. They were also used as thymus donors for mixed thymocyte medium.

2.3.4 SPECIFIC PATHOGEN FREE (SPF) LAMBS

Four SPF lambs, age 4 weeks, born at SPF unit at PSP (Pentlands Science Park). Never exposed or had contact with outside environment. Each lamb was infected with a different cell grown orf virus by skin scarification as previously described (Nettleton *et al.*, 1996). The serological response was measured weakly by ELISA. Statistical analysis of the serological data was done after logistic curves had been fitted to the data for each lamb (Karpinski, 1990). The parameters of the different curves were then compared using t-tests (Watt, 1993).

2.4 PRODUCTION OF MONOCLONAL ANTIBODIES

2.4.1 PREPARATION OF ANTIGEN FOR MAB PRODUCTION

2.4.1a Preparation of purified MRI scab

Using the method of McKeever *et al.*, 1987 approximately 4g of scab was ground in 20mls of PBS. The resulting suspension was clarified by centrifugation at 2000g for 30 minutes at 4°C. The supernatant was divided into two 10mls amounts each of which was layered on to a 3 mls cushion of 36% sucrose (w/w in PBS) before centrifugation at 45,000g for 30 minutes at 4°C using a Beckman SW40ti rotor in a Beckman L2-62B ultracentrifuge. The pellets were each resuspended in 1ml PBS, layered on two 7.5ml continuous gradients (20-60% w/w in PBS) of Nycodenz and ultracentrifuged as before. The viral bands which settled at the middle of the tube were collected from below using a bent needle and syringe and each resuspended in

12ml of PBS before being recovered by ultracentrifugation as above. The pellets of purified virus were then combined and resuspended in 1ml PBS.

2.4.1b Preparation of orf-11 cell-lysate antigen

Two 225 cm² flasks of FLM cells were washed twice with hanks BSS, infected with 2mls per flask of orf-11 virus at a moi of 0.1, and incubated at 37°C for 60 minutes. 100mls of maintenance medium (2% FBS) was added to each flask and incubated at 37°C. At three days post-infection at about 90% cpe the cells were harvested by spinning at 2000g for 15 minutes at 4°C. Cells were washed once in sterile PBS and clarified as before. The cell pellet was resuspended in 2ml of sterile PBS, frozen and thawed three times. The fluid was centrifuged at 12,000g in MSE microfuge for 1 minute. Supernatant was taken and diluted with PBS to 5mls and kept as antigen.

2.4.2 IMMUNISATION SCHEDULE

For the first immunisation equal 1ml volumes of orf antigen and complete Freund's adjuvant were homogenised into a stable emulsion. Mice received 100µl of vaccine subcutaneously in two sites. Three weeks later, the same antigen as used in the first immunisation was mixed with incomplete Freund's adjuvant and a similar dose given as before. Ten weeks after the 2nd vaccination the mice were immunised intravenously with antigen in the tail vein 100µl/mouse.

2.4.3 PREPARATION OF MIXED THYMOCYTE MEDIUM (MTM)

Mixed thymocyte medium (MTM) supernatant was produced to encourage the growth of hybridomas.

MTM: Four rats (two Lister and two Wistar) were killed in a humane way using anaesthetic overdose. Thymuses were collected aseptically into a universal bottle containing RPMI 1640 plus 10% foetal bovine serum (FBS). In the lab the thymuses were ground by a plunger from a 2ml syringe through a metal sieve over a 50ml centrifuge tube. RPMI 1640 plus 10% FBS was added to the pooled thymus suspension and centrifuged at room temperature at 500xg for 5 minutes. Supernatant

was discarded, the pellet was resuspended in 40mls RPMI plus 10% FBS, centrifuged as before and the supernatant decanted off. The pellet was resuspended in 50mls of RPMI plus 10% FBS. Cell viability was demonstrated by trypan blue exclusion and the final cell concentration was adjusted to 5×10^6 /ml. Flasks were seeded with cells and incubated at 37°C in 5% CO₂. Forty-eight hours later supernatant was harvested and clarified by centrifugation at 500xg for 5 minutes. The supernatant was aliquoted in 10ml amounts and stored at -20°C.

2.4.4 FUSION PROTOCOL

The method was similar to that described by Lyaku *et al.*, (1992) with some modifications. Three days after the final boost one of the immunised mice was killed using an anaesthetic overdose. A non-immunised mouse was also euthanised the same way. Blood was collected aseptically by cardiac puncture from immunised and non-immunised mice into different tubes. The spleens were removed into separate labelled universal bottles containing RPMI medium with 10% FBS for the immunised and non-immunised spleen. Each spleen was placed in a separate petri dish. The cells from each spleen were released using two syringes each containing 5mls of medium and injecting the medium into several sites until the capsule became transparent. Spleen cells of immunised and non-immunised mice were each spun for 7 minutes at 500g, pellets were washed once in RPMI 1640 medium containing 10% FBS and once in serum free RPMI 1640 and centrifuged as above. Myeloma cells with >90% viability were washed in the same way.

Immunised spleen and myeloma cell pellets were resuspended in 10mls of serum free RPMI 1640 and non-immunised spleen cells were resuspended in 10% of RPMI 1640 plus 20% FBS. Cell viability counts were obtained using trypan blue and a haemocytometer (Improved Neubauer counting chamber, Weber Scientific International Ltd). A minimum of 5×10^6 Myeloma cells and immunised spleen cells were kept aside as controls. Myeloma and spleen cells were mixed together at a ratio 1:10 and spun down as before. The supernatant was removed and one ml of polyethylene glycol (PEG) 1500 (BDH) was added over 1 minute, while the tube was

gently rotated in order to allow cells to mix. Rotation was extended for another minute and one 1ml of serum free RPMI was added over one minute. An extra 9mls of serum-free RPMI medium was added over 3-5 minutes. After that the cells were pelleted as before and the supernatant discarded. The pellet was gently tapped to resuspend the cells and added to 100mls of RPMI medium with 20% FBS, non-immunised feeder cells at a concentration 2×10^5 /ml and 1x HAT. The final concentration of total cells was 2×10^5 cells/100 μ l. Apart from the peripheral wells 100 μ l of the cell suspension was added to each well of flat-bottom 96-well plates (Nunc, Denmark). A total of 13 plates were set up plus one as control. The control plate contained non-immunised spleen cells, immunised spleen cells and myeloma cells. The spleen cells were included to monitor the secretion of antibody and the myeloma cells were used to observe the potency of the inhibition produced by aminopterin. All the plates were incubated in a humid incubator at 37°C in 5% CO₂. One day post fusion the plates were checked to ensure there was no contamination. Seven days after the fusion the plates were fed with 100 μ l of RPMI containing 15% FBS and 1% hypoxanthine thymidine (HT) and re-incubated for another week meanwhile recording wells that showed cell growth. Hybridomas that grew and covered at least half of the floor of the well were tested for antibody production and cells in any positive wells were subcloned (2.4.6).

2.4.5 SCREENING OF HYBRIDOMAS

2.4.5a Enzyme-linked immunosorbent Assay [ELISA]

2.4.5a₁ Orf-11 ELISA Antigen

The antigen was produced, using a modification of the method of Howard *et al.*, (1985). Briefly, eight 150cm² flasks of confluent FLM cell monolayers were used. Four served as mock controls and the other four were infected with orf-11 virus. The control cells were washed twice with warm hanks medium and 2mls of hanks was added to each flask, while the other four were washed in the same way but infected with 2mls of orf-11 virus, from the same origin of the virus used for the mice immunisation with a titre of $4 \times 10^{6.0}$ TCID₅₀/ml. After an incubation period of 60 minutes 50mls of E199 + 2% FBS was added to each flask and incubated at 37°C.



After the cpe developed to about 90%, the medium from the 4 infected flasks was poured off and clarified in a Beckman centrifuge at 2000g 4°C for 15 minutes. The cell pellets were washed twice with PBS and resuspended in 3mls of PBS+1% NP40 and returned to the flasks. The flasks were kept for 2 hours at +4°C and tilted every 15 minutes. The cell lysates were recovered and clarified in a microcentrifuge (MSE, UK) at 12,000g at room temperature for 5 minutes. The supernatants were pooled together, aliquoted in 100µl amounts in labelled eppendorfs and stored at -70°C. The 4 control flasks were treated in the same way.

2.4.5a₂ Standardisation of the ELISA Ag with 6E11 ascites mab

The optimal working dilution of the orf-11 antigen was determined using a checkerboard titration against anti-orf mab (6E11) ascites kindly provided by I Pow. A serial dilution of the antigen was made in carbonate-bicarbonate buffer pH9.6 and 100µl of each dilution was added to duplicate wells moving down the plate. Negative antigen at the same dilution was also added to duplicate wells. The plate was incubated overnight at 4°C and then washed three times by PBS-T₈₀ and 100µl of 1/200 solution of 6E11 ascites was added to the wells. The plate was then incubated for two hours at room temperature and washed as before. Serial dilutions of sheep anti-mouse immunoglobulin conjugated to horseradish peroxidase (HRP), (Scottish antibody production unit (SAPU)) were made and 100µl was added to appropriate wells across the plate and incubated for two hours at room temperature. After washing, 100µl of 40mg orthophenylene diamine (OPD) (Sigma) per 100ml of citrate phosphate buffer pH5 containing 10µl of 30% hydrogen peroxide (H₂O₂) was added to all the wells. Six minutes later the reaction was stopped by adding 50µl of 2.5M sulphuric acid (H₂SO₄). Results of the optical density (OD) on the wells was read at 492nm in a Titertek Multiskan (Flow laboratories). The optimal working dilution of the antigen was taken as the dilution giving the highest corrected OD (mean of Orf-11 antigen wells minus mean of negative FLM control wells) when the conjugate was used at a dilution of 1/500.

2.4.5a₃ The Assay (indirect ELISA)

Step 1: Plate coating with the virus antigen:

Alternate columns of M129A Dynatech 96-well plates were coated with pretitrated orf-11 lysate and control antigen at the optimum dilution of 1/800 in carbonate-bicarbonate buffer pH9.6 and incubated overnight in a humid box at 4°C. Plates were then washed three times, flooding and emptying with washing buffer PBS-T₈₀ containing 0.1% bovine serum albumin (BSA).

Step 2: Addition of hybridoma supernatant:

Fifty microlitres of neat supernatant fluid from hybridomas was added to one infected and one control well of the antigen-coated plates. Hyperimmune serum (1847), negative SPF serum, anti-orf virus mab supernatant 6E11, and negative mab VPM20 were included as controls. Two hours of incubation at room temperature was conducted, followed by the same method of washing as described previously.

Step 3: Conjugate addition:

One hundred microlitres of predetermined optimal dilution of sheep anti-mouse IgG conjugated to horseradish peroxidase (HRP) (Scottish Antibody Production Unit, Law Hospital, Carlisle, Lanarkshire, UK), diluted in PBS-T₈₀ buffer, containing 1% bovine serum albumin BSA, was added to each well. The incubation period and the washing were repeated as described in Step 2.

Step 4: Substrate addition:

To every well was added 100µl of Orthophenylene diamine (OPD) (Sigma) activated with 30% of hydrogen peroxide (H₂O₂). The reaction was stopped after six minutes by adding 50µl/well of 2.5M of sulphuric acid (H₂SO₄). Optical density (OD) of the test was obtained using Titertek Multiskan ELISA reader at 492nm. Hybridoma supernatants with a corrected OD >0.2 were considered positive.

2.4.5a₄ Immunofluorescent test (IFT)

Any hybridoma supernatant positive by ELISA were also tested by IFT using the method described by Nettleton *et al.*, (1995). Foetal lamb muscle cells were grown on coverslips in tissue culture tubes until nearly confluent. The cells were washed twice with warm hanks BSS. Each tube was infected with 0.2ml of orf-11 virus at a moi of 0.1. Controls had the same volume of hanks and all tubes were incubated at 37°C for 60 minutes after which 1ml of E199 medium, plus 2% FBS was added and cells were incubated at 37°C until the earliest appearance of cpe, usually two days post-infection. The coverslips were washed in PBS and allowed to dry at room temperature. Coverslips were then fixed in neat cold acetone for 10 minutes. Each coverslip was cut into three pieces and adhered to slides by DePex (BDH-England). 50µl of cloned hybridoma supernatant was added to each coverslip and incubated at 37°C for 30 minutes in a moist atmosphere. Coverslips were then washed three times in warm PBS for 5 minutes and allowed to dry as before. The cells were then flooded with 50µl of pretitrated sheep anti-mouse fluorescent isothiocyanate conjugate (FITC), (SAPU) and incubated at 37°C in a moist atmosphere for another 30 minutes. Thereafter the cells were washed three times in warm PBS and dried as before. The coverslips were mounted in glycerol phosphate buffer and examined using a Leitz Ortholux 2 microscope with an ultraviolet light source. Specific fluorescence was seen as bright green staining of the cytoplasm of orf-virus infected cells.

2.4.6 SUBCLONING OF HYBRIDOMAS CELLS

All positive hybridomas were subcloned and limited diluted in 96-well plates to avoid antibody secreting hybridoma cells being overgrown by other cells. Briefly, hybridoma cells recovered from a single colony were subcloned when the cells covered half or more of the bottom of the well. Cells from one well, harvested in 200µl of medium were diluted in 1.8ml RPMI with 10% FBS. RPMI medium containing 10% FBS (100µl) was added to each well of 96-well flat-bottom plates. Starting from the second row of wells a 100µl of the diluted cell was added to each well of the top row and double diluted downwards discarding the last 100µl.

The rest of the cell suspension was transferred as a backup into two wells of a four-well plate (Flow Labs Inc, USA) containing 0.5ml of RPMI medium with 10% FBS. The plates were then covered and incubated at 37°C in 5% CO₂.

2.4.7 STORAGE OF HYBRIDOMAS

Four to five replicates of each secreting hybridoma were frozen down at every stage of the cloning procedure. Each hybridoma was expanded from 96-well plates, to four-well plates, twelve-well plates (Linbro, Flow Laboratories, UK) and finally into 25cm² flasks (Gibco Ltd, Scotland). To make sure that cells being cloned were orf virus antibody secreting cells, hybridoma supernatant was screened at every step from the 96-well to the flask stage. Any weakly positive or negative cells were not passaged further. When the hybridoma cells had grown satisfactorily in the flasks the cells were gently pipetted into 50ml centrifuge tubes and pelleted by spinning down at 500g for 5 minutes. Cell supernatant was collected and stored at 4°C for subsequent studies. The pellet from each flask was resuspended in 1ml of laying away medium, consisting of 40% RPMI, 50% FBS and 10% Dimethyl sulphoxide (DMSO) (Sigma) and transferred into 1.8ml screw-capped cell culture vials. The vials were wrapped in cotton wool and kept at -70°C overnight. On the following day the vials were removed quickly from the cotton wool and stored in the liquid nitrogen.

2.4.8 RECOVERY OF FROZEN HYBRIDOMA CELLS FROM LIQUID NITROGEN

Vials were removed from liquid nitrogen and transferred to a 37°C waterbath. When the cell suspension was just thawed it was removed carefully by pipette and put into a centrifuge tube containing 20ml of RPMI plus 20% FBS and spun down at 500g for 5 minutes. The resulting pellet was resuspended in 4ml of RPMI plus 20% FBS and placed in a twelve-well plate at neat, 1:2, 1:4 and 1:8 dilutions. Plates were incubated in a 5% CO₂ 37°C incubator. Cells growing successfully were tested and expanded as previously described.

2.4.9 PRODUCTION OF ASCITIC FLUID

The strategy was to use a group of five mice for ascites production of each cloned mab. Balb/c mice aged 10-16 weeks were injected intraperitoneally each with 0.5ml of pristane (2, 6, 10, 40 tetramethyl pentadecane (Sigma)). Two weeks later hybridoma cells cloned three times were spun at 500g for 5 minutes at room temperature. Supernatant was removed and stored at 4°C, the pellet resuspended in 2mls of PBS and the cells counted. A total of 5×10^6 cells in 0.2ml of PBS was injected intraperitoneally into each of the pre-pristaned mice which were observed daily. Two to three weeks later when mice developed obvious abdominal swelling ascitic fluid was collected from euthanised mice by tapping from the peritoneal cavity using a 16 gauge needle. The ascitic fluid was centrifuged at 500g for 10 minutes at room temperature. The supernatant fluid was aliquoted and stored at -20°C; sediment which contained cells and debris was discarded.

To investigate the relationship between the ELISA OD values obtained with the mab ascites compared to their third limited dilution of hybridoma supernatant a correlation analysis was carried out (Beyer, 1968).

2.5 CHARACTERISATION OF MONOCLONAL ANTIBODIES

2.5.1 ISOTYPING OF THE MABS

All the monoclonal antibodies were isotyped using the "Sigma" isotyping kit. Each isotyping strip was flooded with 3mls of hybridoma supernatant with gentle agitation and incubated at room temperature for 30 minutes. The strips were then washed three times for 5 minutes with PBS-T₈₀ 0.05% containing 1% BSA. Biotinylated anti-mouse antibody was added for 30 minutes and the strips washed as before. This was followed by 15 minutes incubation in extra avidin peroxidase, three washes again followed by an additional rinse in PBS only. Finally, substrate was added and the reaction stopped quickly in tap water after appearance of the indicator line.

Statistical analysis of the ELISA OD values of the mabs in relation to their isotype was performed using a permutation test (Manly, 1991).

2.5.2 REACTIVITY OF THE MABS AGAINST VACCINIA VIRUS ORF VIRUS RECOMBINANTS BY IFT

A library of 18 recombinant vaccinia viruses expressing various regions of the NZ-2 orf virus genome were used to screen the reactivity of the mabs (Figure 2.1). All the recombinants were produced and kindly supplied by Dr A A Mercer, University of Otago, Dunedin, New Zealand.

Briefly, CV-1 cells were seeded on to coverslips in tissue culture tubes at a concentration 2×10^5 /ml. The cells grew to confluence in 48 hours, after which each tube was infected with 0.2ml of 1:100 dilution of vaccinia virus orf virus recombinant (VVOV). Twenty-four hours later the coverslips were washed in PBS, fixed in cold acetone and treated as described earlier in section 2.4.5a₄ screening of hybridomas.

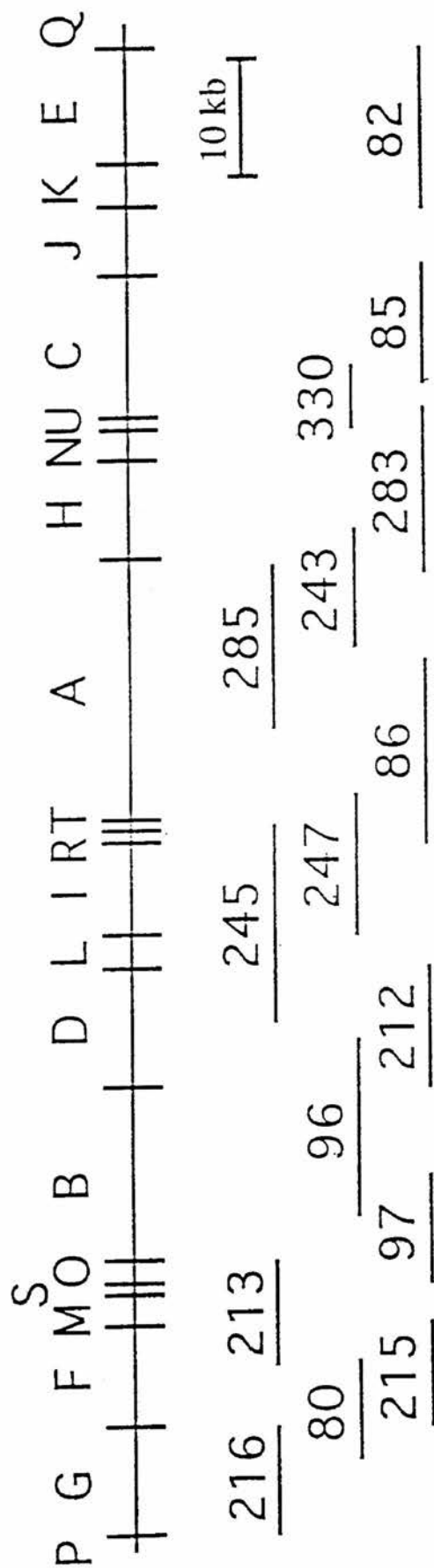
2.5.3 NEUTRALISATION TEST (NT)

Titration of orf-11 for NT:

Using tissue culture grade 96-well plates (Dynatech), 25 μ l of Eagle's 199 (E199) maintenance medium containing 2% FBS was added to all wells in five columns. To each of 5 wells 25 μ l of log₁₀ dilutions of virus was added followed by the addition of 100 μ l of FLM cells in E199 plus 10% FBS at a concentration of 2×10^5 to all wells. The plate was sealed and incubated at 37°C. Virus titre was determined 2-4 days post-infection, using the Spearman-Kärber method. The virus titre was 4.5 log₁₀ TCID₅₀/25 μ l.

Figure 2.1: Map of orf virus NZ2 library underneath site of fragment of the DNA constituting the insertion library. The position of each DNA fragment is shown against the KPN1 map of NZ2 orf strain (Mercer *et al.*, 1997).

Insertion Library Aligned with the *Kpn*I Map of Orf Virus NZ2



The Assay:

The method described by Collins *et al.*, (1984) was followed. To each well apart from the first row 25µl of E199 plus 2% FBS was added. Serial 2-fold dilutions of each ascites was made in duplicate wells down the plate from neat to 64. Control wells also received 25µl of the ascites. Then 25µl of 100 TCID₅₀ of orf-11 virus was added to all the wells except the control and incubated at 37°C for 1 hour. 100µl of FLM cells at a concentration of 2×10^5 cells/ml was added to every well. Plates were sealed and incubated at 37°C for 3 days, when results were read.

2.5.4 WESTERN BLOT

Two antigens were used (a) purified antigen and (b) cell-lysate antigen.

2.5.4a Purified antigen

Ten 225cm² flasks of newly confluent FLM cells were washed twice with hanks, before each flask was infected with orf-11 virus at a moi of 0.1. The virus was allowed to adsorb for 60 minutes at 37°C. Four days post-infection when the cpe was about 95% the flasks were frozen and thawed three times and the medium spun down at 2000xg 4°C for 15 minutes (Beckman). The supernatant was dispensed in Kontron 45 (K45) tubes and centrifuged at 169,800xg for 60 minutes at 4°C in a Beckman XL-70 ultracentrifuge. Supernatant was carefully removed, the pooled virus pellets resuspended in 9mls of supernatant and transferred into SW40ti Beckman centrifuge tubes containing 3mls of 36% sucrose in PBS. The tubes were spun at 71,000g for 30 minutes, after which the supernatant was gently aspirated and the pellet resuspended in 1ml of sterile PBS. A final centrifugation was made after loading the resuspended pellet on a 20-60% sucrose gradient in SW40ti tubes and centrifuged at 71,000g for 30 minutes. Sixteen fractions of antigen were collected in bijoux bottles after piercing the bottom of the tube. Titration of all fractions was performed, and five peak fractions of virus infectivity were pooled and the protein concentration measured.

2.5.4b Cell-lysate antigen

Four 225cm² flasks of FLM cells were infected with the same orf-11 virus as described in 2.5.4a above. Four days post-infection, medium was poured into 50mls centrifuge tubes, adherent cells were scraped off and added to the medium which was clarified at 2000xg for 10 minutes. Cell pellets were washed once in 20mls (PBS) and clarified as before. The pelleted cells were raised in 2ml of PBS and frozen and thawed three times. The suspension was centrifuged in a high speed microfuge (MSE) at 12,000g for 1 minute and the supernatant aliquoted in 200µl and stored at -20°C until used. Four mock-infected flasks were treated similarly alongside the infected flasks to provide control cell lysate antigen.

2.5.4c Total protein determination

The total protein in the purified orf antigen was measured by the Pierce method using a BSA protein assay reagent kit from Pierce and Warriner (UK) Ltd. Solutions containing a known concentration of bovine serum albumin (BSA) were used as standards. Results were read at 550nm expressed as ug/ml and printed out from a computer interfaced to a Titertek Multiskan plus.

2.5.4d Blotting test

The method of McKeever *et al.*, (1987) was used with a modified non-radioactive protein staining method. Briefly, equal volumes of the antigen containing 6185.7µg/ml of protein and loading buffer were mixed and boiled for 5 minutes. Treated antigen was loaded on to an 11% discontinuous polymerised polyacrylamide gel in a Biorad Mini Protean II dual slab gel apparatus alongside marker proteins. The gel was run at 150V for 30-45 minutes until the dye reached the bottom of the gel. The proteins were transferred on to nitrocellulose membrane for an hour at 100V.

Bands of protein in nitrocellulose membrane were visualised after staining with 0.2% ponceau red for 2-5 minutes. The stain was removed by washing three times in PBS Tween₈₀ (0.05%). The nitrocellulose was then blocked in 1 x PBS Tween₈₀ (0.05%)

for an hour at room temperature. The blocking and the washing were carried out with gentle mixing. The nitrocellulose was cut into 5mm wide strips, and each strip was incubated in a neat hybridoma supernatant or a predetermined concentration of 1:200 of each monoclonal antibody ascites or 1:100 of 1847 sheep anti-orf hyper-immune serum for an hour at room temperature and washed. Pretitrated horseradish peroxidase HRP conjugated sheep/anti-mouse IgG or donkey anti-sheep/goat IgG (Sapu) was added for an hour at room temperature. The strips washed again and 3,3 diaminobenzidine tetrahydrachloride (DAB) at a concentration of 5mg in 10mls of 10mM Tris Hcl with 200µl of 30% H₂O₂ was added for 2-5 minutes. When the colour developed the strips were rinsed quickly in tap water and allowed to dry. Molecular weights of protein in the nitrocellulose were determined from a standard curve measured from the marker proteins against their known molecular weight on a semi-logarithmic scale (Shapiro *et al.*, 1967).

2.5.5 RADIOIMMUNOPRECIPITATION (RIP)

Orf-11:

Orf-11 was concentrated 100X by ultracentrifugation using the Kontron K45 rotor. The titre of the concentrated virus was $4 \times 10^{8.0}$ TCID₅₀/ml.

FLM cells:

Foetal lamb muscle cells were seeded into 3.5cm² petri dishes at a concentration of 5×10^5 cells in 1ml. They were used when confluent 2 days later.

RIP test:

The method of Sullivan *et al.*, (1994) was employed with some modifications. Basically, 10 petri dishes were washed with warm PBS twice, 9 of which were infected with concentrated orf-11, using a moi of 10. After an adsorption time of 60 minutes, 1ml of E199 medium was added to each petri dish and incubated at 37°C. Thirty-six hours later the medium was aspirated and the cell sheet washed twice with warm PBS. One ml of methionine free RPMI 1640 medium (plus 0.2ml supplement of Arginine, cysteine, leucine, inositol and glucose) was added to each

petri dish and incubated for 30 minutes at 37°C in 5% CO₂ after which 25µl of labelling ³⁵S methionine (Amersham, UK) was added to each of the petri dishes and incubated as before for 3 hours tilting every 30 minutes. Thereafter, the medium was discarded into Decon, and the cell sheet washed twice with cold PBS. Cells were lysed for 20 minutes following the addition of 250µl of cold RIPA buffer, containing 2.5µl of 10mg/ml aprotinin and 2.5ml of 10mg/ml phenylmethylsulfonyl fluoride (PMSF) to minimise proteolytic activity in the cell extract.

The cells and the extract were transferred into 1.5ml eppendorfs and centrifuged at 11,600g for 2 minutes. The supernatant was transferred to fresh screw-capped eppendorf tubes and used immediately or stored at -20°C.

Precipitation:

Four microlitres of anti-orf hyper-immune serum (1847) or 1 to 2µl of ascitic fluid was added to 125µl of each extracted supernatant mixed gently and incubated overnight at 4°C. Extracts of uninfected cells reacted with antibody and one extract of infected cells without added serum or ascites fluid were also included. Twenty microlitres of pre-soaked and washed protein G agarose (10mg/ml) (Sigma) was added to the immunocomplexes and incubated for approximately 7 hours at 4°C, mixing from time to time. Fifty microlitres of RIPA buffer was then added with gentle agitation and then the tubes were spun at 11,600g for 2 minutes to precipitate the immunocomplexes. The supernatant was discarded into Decon, and each pellet was resuspended in 500µl RIPA buffer and washed twice on ice and clarified as above. To denature the protein each pellet was resuspended in 40µl of 1 x loading buffer, boiled for 5 minutes, and clarified at 11,600g for 2 minutes. Fifteen microlitres of supernatant from each sample was loaded into a well on a 3% stacking gel over a 15% SDS PAGE gel (Biorad Protean II slab gel) and 4.5µl of ¹⁴C radiolabelled rainbow molecular weight markers (Amersham) were also included in a separate well. Gels were run at 80V overnight and then transferred on to blotting paper and dried in a slab gel dryer (Hoefer Scientific Instruments, San Francisco) for at least 90 minutes. The gel was covered with x-ray film (Fuji Film, Japan) and

stored in a cassette for 7 days. Film was developed after one week after which a second film was put in the cassette and this was developed 6 weeks later.

Following determination of the size of proteins detected by each mab the ELISA OD values of the two major groups of mabs were compared statistically using a t-test (Watt, 1993).

2.5.6 PULSE-CHASE EXPERIMENT

A modified method of Laemmli (1970) and Sullivan *et al.*, (1994) was conducted. Briefly, petri dishes were set up as described previously for RIP. All plates were infected at the same period. Isotope labelling was 1 hour for each sample and one plate was harvested every three hours. For precipitation each sample was divided into two, one was mixed with 2E5 (an anti 40kd) mab and the other with 6E2 (an anti 65kd mab). The rest of the procedure was as described for RIP.

2.6 REACTIVITY OF MONOCLONAL ANTIBODIES WITH OTHER PARAPOXVIRUSES (IFT & ELISA)

2.6.1 PREPARATION OF COVER SLIPS AND IFT STAINING OF THE OTHER PARAPOXVIRUSES

Apart from the time of cell fixation, which depended on the appearance of cpe, the method was the same as described previously in section 2.4.5.

2.6.2 PRODUCTION AND STANDARDISATION OF ELISA ANTIGEN FROM THE SAME PPVS AS IN 2.6.1

Four 225cm² flasks of FLM were used Two were washed and mock-infected as controls while two were each infected with the appropriate irus at an moi of approximately 0.1. Detergent lysates of control and infected cells were prepared as previously described in section 2.4.5.

Standardisation of ELISA Antigens:

To ensure reactivity of each ELISA antigen two plates were set up. Starting with an antigen dilution of 1/50, 100µl of serially diluted (two-fold) mock and test antigen were coated on to alternate columns of a 96-well plate and incubated at 4°C overnight. Following washing, 100µl of a monoclonal, previously known to react by IFT and to give a high OD in the Orf-11 ELISA was added to one plate, at 1:200 dilution. After two hours at room temperature incubation period and the usual wash, 100µl of two-fold dilutions (from 1/500 to 1/4000) of sheep anti-mouse HRP conjugate (SAPU) was added to each well in duplicate rows and incubated again for two hours. This was followed with proper wash and addition of the opd substrate. After six minutes, the reaction was stopped by adding 50µl of 2.5M sulphuric acid. With all antigens the optimum anti-mouse HRP conjugate dilution was 1/500.

To the second plate, coated with the same antigen as described above, hyperimmune anti-orf virus serum (1847) and SPF serum were used at dilutions of 1/1000 instead of mabs and Donkey anti-sheep conjugate dilution were used instead of sheep anti-mouse conjugate. Similar method was used to standardise all other parapoxvirus.

2.6.3 COMPARATIVE PARAPOXVIRUS ELISA ASSAY

Eight M129A Dynatec plates were used and each was coated with a separate ppv ELISA antigen (orf-11, NZ2, D1701, scabbymouth, Milker's Node, bovine papular stomatitis, seal parapox and squirrel parapox) and FLM control antigen in carbonate-bicarbonate buffer. The plates were then incubated overnight at 4°C.

Each of the 27 ascites was diluted in bijou 1:200 in the diluent buffer containing (1xPBS-T₈₀, 1% BSA). 100µl of each mab was added to positive and negative wells in each of the eight plates. Hyper-immune serum (1847), negative SPF serum and negative mab VPM20 were included as controls.

All the washing, incubation periods and other procedures were as described in screening of hybridomas.

Correlation analysis was performed using Spearman Rank correlation on the mab ELISA OD values with those antigens that produced good binding with the polyclonal hyperimmune anti-orf virus serum (Siegal, 1956).

2.7 DEVELOPMENT OF MAB BASED VIRUS DETECTION METHOD

2.7.1 CONJUGATION OF MONOCLONALS TO HORSERADISH PEROXIDASE (HRP)

Eight Mabs were selected and the protein concentration of their ascites was measured as described by Jones *et al.*, (1989).

2.7.1a Affinity chromatography

The method used, was as given by Pharmacia in their monoclonal antibody purification handbook. The column was packed with protein G sepharose 4 fast flow. The column was run at room temperature in PBS at a flow rate of 0.5ml/min. Samples were applied to the column and unbound protein washed off using PBS until no protein was detected in the effluent by UV at 280nm. The column was then eluted with 0.1M Glycerine-HCL pH2.7.

2.7.1b Conjugation method

The method used was as described by Wilson and Nakane (1978). Basically, 4mg horseradish peroxidase (Sigma type VI P8375) was dissolved in 1ml distilled water. 0.2mls of freshly prepared 0.1M Sodium Iodate was added and stirred for 20 minutes at room temperature. The resulting HRP0-aldehyde solution was dialysed overnight at 4°C, against 1mM sodium acetate buffer pH4.4. After that the HRP0-aldehyde was adjusted to pH9.5 by adding 0.5M sodium carbonate, followed by the immediate addition of 8mg immunoglobulin in 1ml 0.01M sodium carbonate buffer pH9.5 and stirred for 2 hours at room temperature. 0.1ml of freshly prepared sodium borohydride solution (4mg/ml in distilled water) was added and stored for two hours at 4°C. After that the mixture was chromatographed on 1.6x90cm sephadex G100 column in PBS. The flow rate was approximately 12ml/hr and 2ml fractions were collected.

Absorbance of the fractions was measured at 280nm and 403nm. Fractions with an OD₄₀₃/OD₂₈₀ ratio of approximately 0.3-0.6 and an OD₂₈₀ > 0.1 were pooled together, BSA was added at 10mg/ml, after which the conjugated were aliquoted and stored at -20°C. (The HRP conjugation was kindly done by D Harkins).

2.7.2 DETERMINATION OF THE ACTIVITY OF THE HRP-CONJUGATED MABS

Plates were coated as before with the previously determined optimal dilution of orf-11 and mock ELISA antigens overnight at 4°C. After washing, doubling dilutions of the conjugated mabs, starting from 1:200 in (1% bovine albumin in 0.05% PBS-Tween₈₀) were added to single wells. Plates were then incubated for two hours at room temperature followed by washing and the addition of substrate. Six minutes later the colour was stopped by addition of sulphuric acid, plates were then read in the ELISA reader as described before. The end point dilution of each conjugate was determined as the highest dilution with a corrected OD \geq 0.2.

2.7.3 COMPETITION ELISA

Step 1: Coating the plates:

The method of Saliki *et al.*, (1993) was followed. Briefly sixteen ELISA plates were set up, and alternate columns coated with previously optimised orf-11 and FLM ELISA antigens. Plates were incubated at +4°C overnight as described in section 2.4.5.

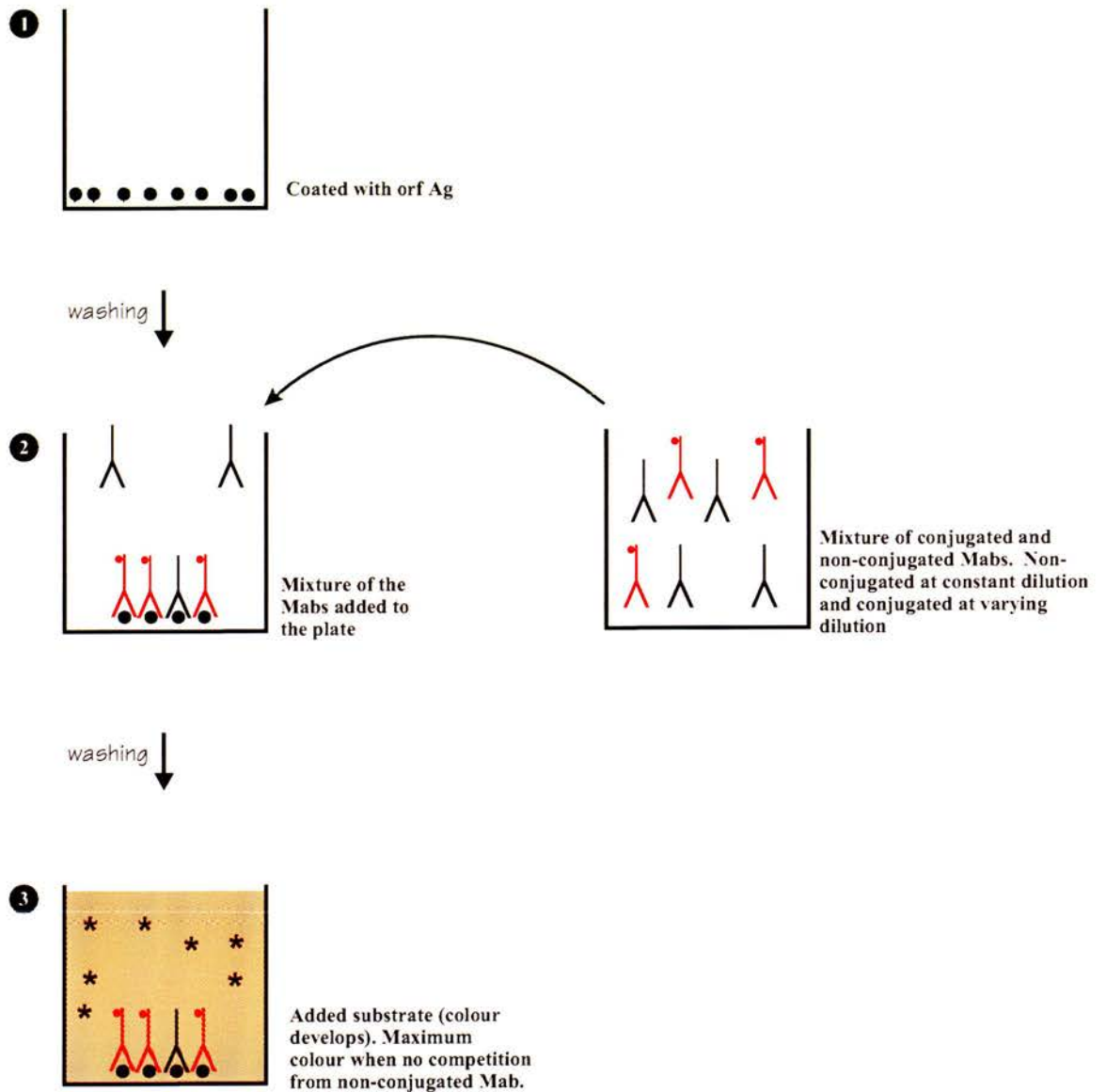
Step 2: Preparation and addition of mixtures of mabs:

In separate (Transfer) plates (Figure 2.2), a previously determined optimal dilution of constant un-conjugated test mabs were mixed with variable ten-fold dilutions (200; 2000; 20,000; 200,000) of horseradish-peroxidase (HRP) conjugated mabs. One hundred microlitres of each mixture was added to each well in the top four rows of the ELISA plates. To each well in the bottom four rows 100µl of a mixture of constant anti-pestivirus VPM20 mab, used at the same dilution as non-conjugated mab, and 100µl of conjugated mabs of the same dilution used for the top row was added. Concentration of the unconjugated mabs was determined from a previous

Figure 2.2: Schematic diagram of ELISA used to demonstrate conjugated and non-conjugated monoclonal antibodies competing for binding sites on orf viral antigen. Step 3 shows development of colour after addition of the substrate.

Elisa plate

Transfer plate



titration, which was chosen to have an OD reading approximately equal to 1.00. The plates were then incubated overnight at 4°C.

Step 3: Addition of the substrate:

After the usual wash, 100µl of the substrate was added to each well, and the reaction was stopped as described earlier.

Evidence of competition was determined by comparison of the OD reading of the unconjugated + HRP conjugated mabs with the mock VPM20 + HRP conjugated mabs at each dilution.

2.7.4 CAPTURE ELISA

2.7.4a Determination of ability of mabs to capture and detect orf virus antigen

Eight HRP-conjugated mabs and their homologous non-conjugated mabs were selected and tested based on the method of Fenton *et al.*, (1991). Generally each of the non-conjugated mabs (6E2, 6E8, 7C9, 8B6, 8G5, 9C3, 10E6 and 13C10) were diluted 1:1000 in carbonate-bicarbonate buffer and 100µl was added to each well. Plates were then incubated at 4°C overnight. After washing, 100µl of pre-determined FLM antigen and orf-11 ELISA antigen were added to the appropriate wells for 2 hours at room temperature. Thereafter, plates were washed again and 100µl of 1:1000 of the HRP-conjugated mabs was added to the appropriate wells. Addition of substrate, stop solution and reading were as described earlier in section 2.4.5.

2.7.4b Optimisation of capture and detector mabs

To optimise the titre of each mab combination, checkerboard titration were performed. Serial dilutions (1:1000; 1:5000; 1:10,000) of capture non-conjugated mabs was carried out in coating buffer and added to ELISA plates. All the incubation and washings were as described in section 2.7.4a. Constant concentrations of the pre-titrated orf-11 and FLM antigens were added to the wells. Subsequently, HRP-conjugated mabs diluted in the same series were added to the appropriate wells,

and the substrate addition, stopping and reading of the results were performed as described previously (section 2.4.5).

2.7.4c Capture ELISA using orf virus ELISA antigen

Step 1: Coating the plates:

The technique of Fenton *et al.*, (1991) was followed. Four combinations of capture and detector mabs were used. Capture mabs were diluted in carbonate/ bicarbonate buffer pH9.6, 100µl was added to the plates and kept at 4°C overnight.

Step 2: Addition of orf-11 and FLM antigen:

After plates had been washed three times, 100µl of pre-determined orf-11 and FLM ELISA antigens were added to the appropriate wells. Plates were then incubated for 2 hours at room temperature and washed as before (section 2.7.4b).

Step 3: Addition of detector mabs:

100µl of previously titrated HRP-conjugated mabs (detector mabs) was added to the appropriate wells. The plates were incubated for 2 hours at room temperature and washed afterwards with washing buffer (Figure 2.3).

Step 4: Substrate addition:

Activated orthophenylene diamine (opd) was added to all the wells. The reaction was stopped and read as described in section 2.4.5.

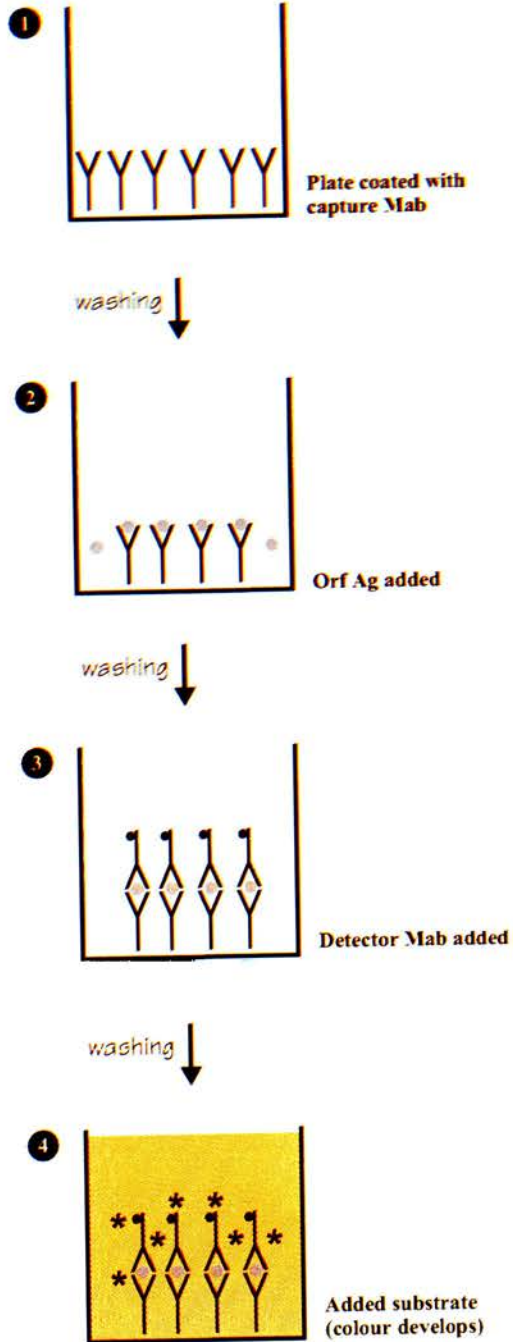
The ELISA OD values of the mab capture/detector combinations with the 4 different virus were compared using analysis of variance (ANOVA) (Watt, 1993).

2.7.4d Capture ELISA of orf tissue culture grown virus

Except for the dilutions of the tissue culture grown virus used (Neat, 1:10; 1:100; 1:1000) the test procedure was identical to the capture ELISA using the orf-11 ELISA antigen.

Figure 2.3: Schematic diagram of capture ELISA used to detect orf virus antigen in biological specimens. Non-conjugated mab was used to capture Ag followed by the addition of conjugated mab as detector. Step 4 shows development of colour after addition of the substrate.

Elisa plate



Statistical analysis was done on the four tables (6.7a-6.7d) representing capture of tissue culture grown virus by applying co-variance analysis (Mead, 1990).

2.7.4e Capture ELISA for demonstrating orf virus antigen in scabs

Preparation of scab antigen for capture ELISA:

Two preparation methods were used:

Method 1: Purified orf antigens from scab. As before one gram of scab was mixed with sterile sand and 10ml of PBS and ground thoroughly. The emulsion was centrifuged at 2000g for 30 minutes at 4°C. The supernatant was removed and layered on 3ml of 36% sucrose (w/w) in PBS and spun in Beckman SW40ti tubes at 71,000g for 30 minutes. The supernatant was discarded into disinfectant (FAM). The pellet was resuspended in 1ml of sterile PBS and 9mls of PBS was then added and centrifuged again in 10ml of PBS SW40ti tubes at 71,000g for 30 minutes. The supernatant was discarded and the pellet resuspended in 1ml sterile PBS. 30µl of the suspension was aliquoted in eppendorf for Electron microscopy (sample 1). To the rest 10µl of NP40 was added and incubated at 37°C for 2 hours and mixed every 30 minutes. After that another 30µl of suspension was removed for EM examination (sample 2).

Method 2: One gram of orf scabs was ground thoroughly in 10ml of PBS and spun at 2000g (Beckman) for 15 minutes. The supernatant was collected, 100µl of NP40 (Sigma) was added and the mixture incubated at 37°C for 2 hours with tilting every 30 minutes.

Capture ELISA:

The method performed was no different from the capture ELISA of orf ELISA antigen and the capture ELISA using tissue culture grown virus. The scab capture ELISA was set up using the scab antigen at varying dilutions with SPF (Negative serum) and 1847 hyperimmune serum as capture antibody and 6E8 HRP conjugated mab as detector.

CHAPTER 3
PRODUCTION OF MONOCLONAL ANTIBODIES

3.1 INTRODUCTION

Monoclonal antibodies contain only a single type of antibody molecule produced by hybridoma cells which have been cloned from a single ancestor cell (Roitt *et al.*, 1985).

In the mid-seventies Kohler and Milstein (1975) were the first to demonstrate the successful production of mabs. The mabs they produced reacted specifically against sheep red blood cells. Since that time many other researchers have employed the technique and mab production has become an important technology for producing specific reagents against protein epitopes (Collins *et al.*, 1984; Morrison *et al.*, 1985; Kitamoto *et al.*, 1986a; Highlander *et al.*, 1987; Kitamoto *et al.*, 1987; Czerny and Mahnel, 1990; Lyaku *et al.*, 1992).

The general principle and practice which has now become established is to use a myeloma cell line, which grows in culture but lacks hypoxanthine phosphoribosyl transferase (HGPRT), as a fusion partner with spleen cells of immunised animals. After fusion, and to obtain only hybridoma cells and to remove unfused myeloma cells, the cells are grown in selective medium supplemented with hypoxanthine, aminopterin and thymidine (HAT). The aminopterin of the HAT medium will block the pathway of synthesis of cellular DNA, in addition, the alternative or salvage pathway cannot take place in the myeloma cells due to their deficiency in HGPRT. For these two reasons the myeloma cells die. Therefore only the fused myeloma and spleen cells survive in the HAT medium where the spleen cells supply functional HGPRT enzyme and the myeloma cells supply the ability to grow in tissue culture. The hybridomas grow continuously in culture medium, and are subsequently screened for specific antibody production.

So far, only two studies have been published on orf virus mabs, one in USA and the other in Germany (Lard *et al.*, 1991; Czerny *et al.*, 1997). A third study has been done in France and presented in a doctoral thesis (Schindler, 1995).

One of the great advantages of mabs is that they have improved dramatically the usefulness of immunoassays and the reliability of serological tests (Harlow and Lane, 1988).

This chapter describes the production of monoclonal antibodies against orf virus following the use of two different orf virus preparations for the immunisation of mice.

3.2 RESULTS

3.2.1 HYBRIDOMAS FROM AN EARLIER FUSION

In a study carried out previously a number of orf virus specific hybridomas were produced but the resulting mabs had been incompletely characterised. This fusion had used virus purified from orf scabs and up to 21 anti-orf virus-secreting hybridomas had been successfully cloned with 10 being used for ascitic fluid production. One (5C8) had been shown to be useful for the immunostaining of orf antigen in infected skin (Jenkinson *et al.*, 1992) but otherwise the only other available information was that they had all reacted with an orf virus protein of approximately 40kDa by western blotting. Five hybridomas including 5C8 were raised and tested. Unfortunately the 5C8 hybridoma cells failed to grow but three grew successfully. One produced weak antibody and was not used further, but two (2E5 and 6E11) were recloned and used for further studies. Availability of these hybridomas allowed optimisation of the ELISA screening method to be used in the subsequent fusion. Dilution of the orf virus ELISA antigen showed that at low dilution the ELISA OD value was reduced possibly due to an excess of non-specific protein or detergent and that the optimal dilution of the ELISA antigen was 1/800 (Fig 3.1).

3.2.2 MAB PRODUCTION USING CELL LYSATE ANTIGEN

From the second fusion which used spleen cells from mice immunised with a lysate of Orf-11 infected cells, thirteen 96-well plates were set up. Hybridomas grew in 293 wells. All the 293 hybridomas were screened by ELISA at least twice. Forty-one of

the 293 were found to secrete anti-orf virus antibodies and twenty-five of the forty-one were successfully cloned and used to produce ascites. The rest of the hybridomas were not cloned because of overgrowth with non-secreting hybridomas, loss of their ability to secrete specific antibodies or death.

3.2.2.a Screening by ELISA

Hybridomas were tested when the cells covered half the floor of the well or more, which means they were screened at different times. The ELISA OD values obtained for the hybridoma supernatants between passages from the original cell colony through to the third limiting dilution was variable (Table 3.1). Because 2E5 (1st fusion) and 6E11 had been cloned using an IFT screening method, only the ODs of the 25 hybridomas cloned from the second fusion were recorded. By the time they had been cloned three times, fourteen mabs showed a decrease of >0.2 of their OD reading (1C7, 1D9, 1G5, 2B5, 3C7, 3F5, 5B5, 5C3, 6E2, 8D7, 10E6, 11B6, 11D7, 13E4) Seven mabs revealed little change (<0.2) in their OD values (2B4, 2B6, 6C8, 6E8, 8B6, 8G5, 11E4) while only four mabs showed an increase in OD value (2E5 (2nd fusion), 7C9, 9C3, 13C10). (Table 3.1).

3.2.2.b Confirmation of mab reactivity by IFT

Supernatant cell culture fluids from all the hybridomas which were found positive by ELISA were tested by IFT using orf-11 virus infected FLM cells. All 27 mabs were clearly positive, confirming the efficiency of the ELISA for detecting anti-orf virus mabs. Two types of fluorescent distribution were observed in IFT. All mabs gave diffuse cytoplasmic fluorescence of the infected cells, indicating the site of viral multiplication, while two mabs (5C3 and 11D7) also gave dense fluorescent staining around the cell membrane. These patterns of reactivity are described in more detail in Chapter 5.

Figure 3.1: Typical titration curve of orf-11 ELISA antigen against 1/200 dilution of ascites (6E11), and anti-mouse HRP conjugate. Each optical density reading represents the average of two replicates at (OD_{492nm}).

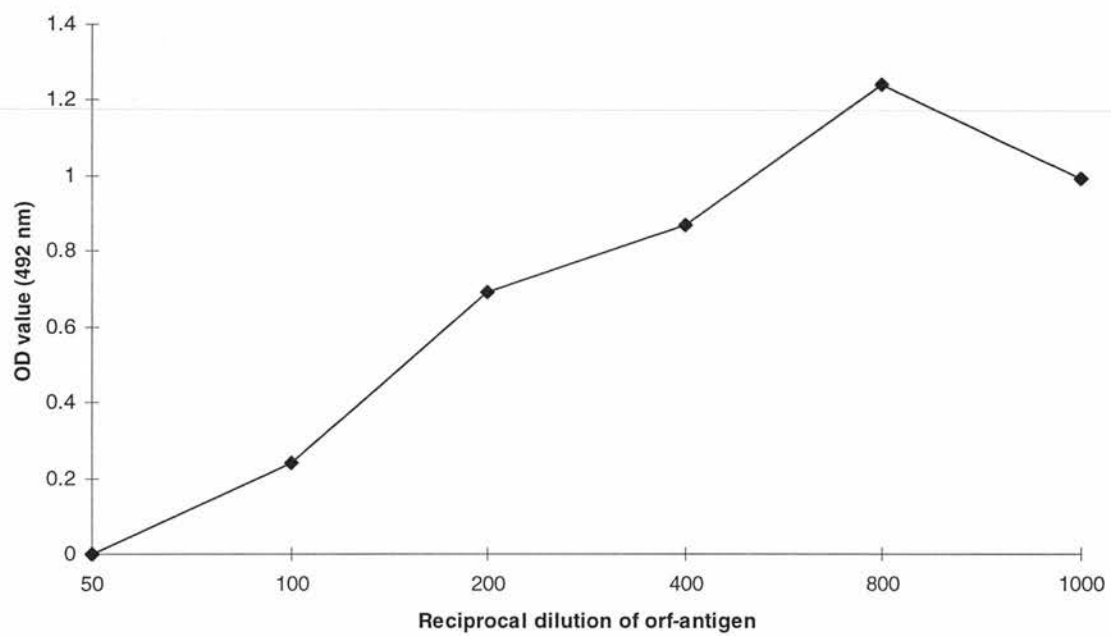


Table 3:1 ELISA record of hybridoma supernatant fluids from original cell colony through the cloning stages

	Mab designation	OD of the original well	OD of First limiting dilution	OD of Second limiting dilution	OD of Third limiting dilution
1.	1C7	1.34	0.61	0.56	0.59
2.	1D9	0.99	0.65	0.40	0.57
3.	1G5	0.69	0.40	0.66	0.37
4.	2B4	0.59	0.55	0.67	0.51
5.	2B5	2.09	0.24	0.56	0.61
6.	2B6	0.51	0.62	0.87	0.48
7.	2E5(second fusion)	0.48	0.90	0.31	0.94
8.	2E5 (first fusion)	(IFT only)	(IFT only)	(IFT only)	2.45
9.	3C7	0.94	0.53	0.33	0.35
10.	3F5	0.94	0.45	0.25	0.36
11.	5B5	2.30	0.51	0.10	0.44
12.	5C3	1.09	1.29	0.88	0.67
13.	6C8	0.41	0.23	0.40	0.46
14.	6E2	1.20	0.36	0.75	0.85
15.	6E8	2.37	1.96	2.00	2.47
16.	6E11	(IFT only)	(IFT only)	(IFT only)	0.79
17.	7C9	0.71	1.06	1.24	0.93
18.	8B6	1.13	0.75	0.42	1.22
19.	8D7	2.00	0.97	1.34	1.13
20.	8G5	0.76	0.43	0.99	0.88
21.	9C3	1.61	2.45	2.47	2.48
22.	10E6	1.23	1.47	0.82	0.76
23.	11B6	0.67	0.53	0.77	0.44
24.	11D7	0.62	0.63	0.40	0.20
25.	11E4	0.45	0.61	0.58	0.49
26.	13C10	0.68	2.26	1.98	2.02
27.	13E4	0.63	0.83	0.51	0.42
28.	+ve control (6E11)	0.42	0.67	0.85	0.44
29.	-ve control	0.03	0.10	0.07	0.05

3.2.3 ISOTYPING OF HYBRIDOMA SUPERNATANTS

Isotyping of the 27 cloned hybridoma supernatants showed that 13 were IgG1, four were IgG2a and five hybridomas secreted IgG2b. One was found to yield IgG3 (10E6), one produced IgM (2B6) and three mabs (1D9, 3F5, 11D7) were IgA class immunoglobulin (Table 3.2).

There was no obvious association between the strength of OD value in the ELISA and the class of immunoglobulin of the hybridomas, except that mabs of IgA isotype had lower ODs. When the ELISA OD values of the mab supernatants were grouped by isotype (Table 3.3a) and statistically analysed using a permutation test the IgA ELISA values ($\bar{x}=0.377$) were significantly lower ($P=0.03$) than those of the combined IgG1 and IgG2 isotypes ($\bar{x}=0.978$). There was no significance difference between the ELISA values of the IgG1 and IgG2 isotypes. A similar and confirmatory result was obtained when the ELISA OD values of the mabs ascites were grouped and analysed by the same method (Table 3.3b). Again the IgA values ($\bar{x}=0.250$) were significantly lower ($P=0.01$) than those of the combined IgG1 and IgG2 isotypes ($\bar{x}=0.711$). There was no significant difference between the ELISA OD values of the IgG1 and IgG2 isotypes.

Table 3:2 Immunoglobulin isotype secreted by hybridomas

	Mab designation	IgG1	IgG2a	IgG2b	IgG3	IgM	IgA
1.	1C7	+					
2.	1D9						+
3.	1G5	+					
4.	2B4	+					
5.	2B5	+					
6.	2B6					+	
7.	2E5(second fusion)		+				
8.	2E5 (first fusion)	+					
9.	3C7		+				
10.	3F5						+
11.	5B5			+			
12.	5C3	+					
13.	6C8			+			
14.	6E2			+			
15.	6E8		+				
16.	6E11		+				
17.	7C9	+					
18.	8B6	+					
19.	8D7			+			
20.	8G5	+					
21.	9C3			+			
22.	10E6				+		
23.	11B6	+					
24.	11D7						+
25.	11E4	+					
26.	13C10	+					
27.	13E4	+					

Table 3.3a ELISA ODs of the third limiting dilution of each mab hybridoma cell supernatant according to its isotype.

	IgG1	IgG2a	IgG2b	IgG3	IgM	IgA
	2.45	2.47	2.48	0.76	0.48	0.57
	2.02	0.94	1.13			0.36
	1.22	0.79	0.85			0.20
	0.93	0.35	0.46			
	0.88		0.44			
	0.67					
	0.61					
	0.59					
	0.51					
	0.49					
	0.44					
	0.42					
	0.37					
n	13	4	5	1	1	3

Table 3.3b ELISA ODs of 1/200 dilution of mab ascites according to their isotype.

	IgG1	IgG2a	IgG2b	IgG3	IgM	IgA
	1.79	1.99	1.40	1.02	0.59	0.39
	1.31	0.50	1.23			0.21
	0.70	0.38	0.61			0.15
	0.68	0.35	0.49			
	0.60		0.30			
	0.57					
	0.52					
	0.47					
	0.45					
	0.38					
	0.35					
	0.29					
	0.27					
n	13	4	5	1	1	3

3.2.4 ASCITES PRODUCTION AND TESTING

Ascitic fluid was successfully obtained from all the mabs produced. Individual hybridomas had different growth characteristics with some clones growing faster than others. For example, mabs 6C8, 6E2, 6E8, 9C3, 10E6 and 13C10 multiplied much faster than 1G5, 5C3, 5B5. In general, however, ascitic fluid developed two to three weeks following injection of the hybridoma cells into mice. Instead of production of ascitic fluid some mice which had been injected with hybridoma cells developed solid tumours. Three mice injected with 5B5, two mice inoculated with 8D7, one mouse with 5C3, another with 6E8, one with 10E6 and one with 11B6 developed solid tumours.

When the ascitic fluids of the 27 mabs were screened at a 1/200 dilution in an indirect ELISA all were positive with the OD values varying between 0.15 and 1.99 (Figure 3.2). The OD value of a 1/200 dilution of the anti-BVD virus mab VPM20 in the same test was 0.04

3.2.5 RANKING OF THE MAB ASCITES BY ELISA AND IFT

Ranking of the mabs according to the ELISA OD values of their ascites revealed six mabs (6E8, 2E5 (first fusion), 9C3, 13C10, 8D7, 10E6) with ODs >1.0. Eleven mabs had OD values <1.0 to >0.4 OD and the other 10 mabs scored <0.4 to 0.15 (Table 3.4).

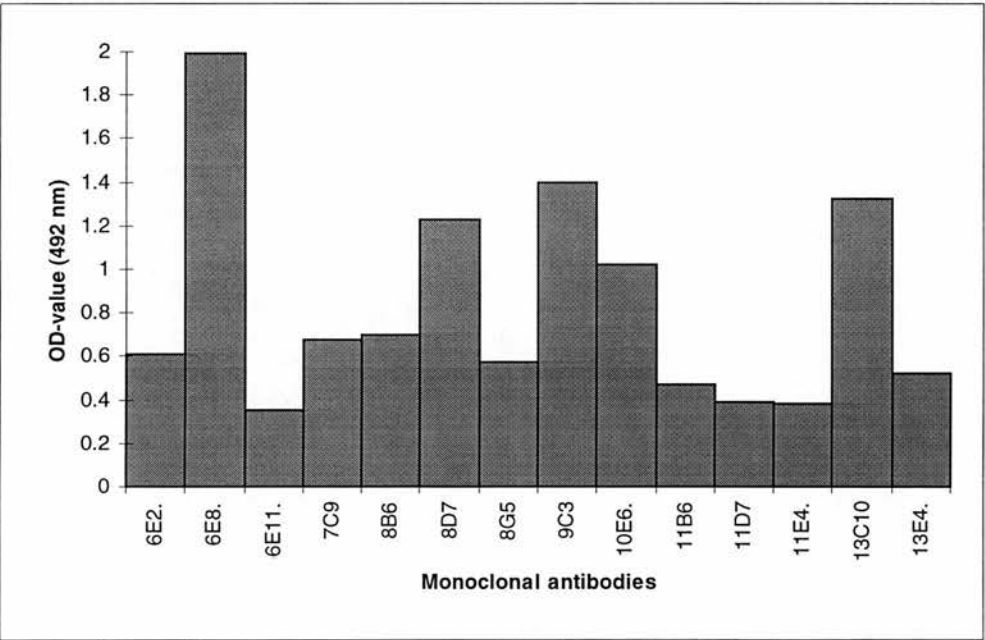
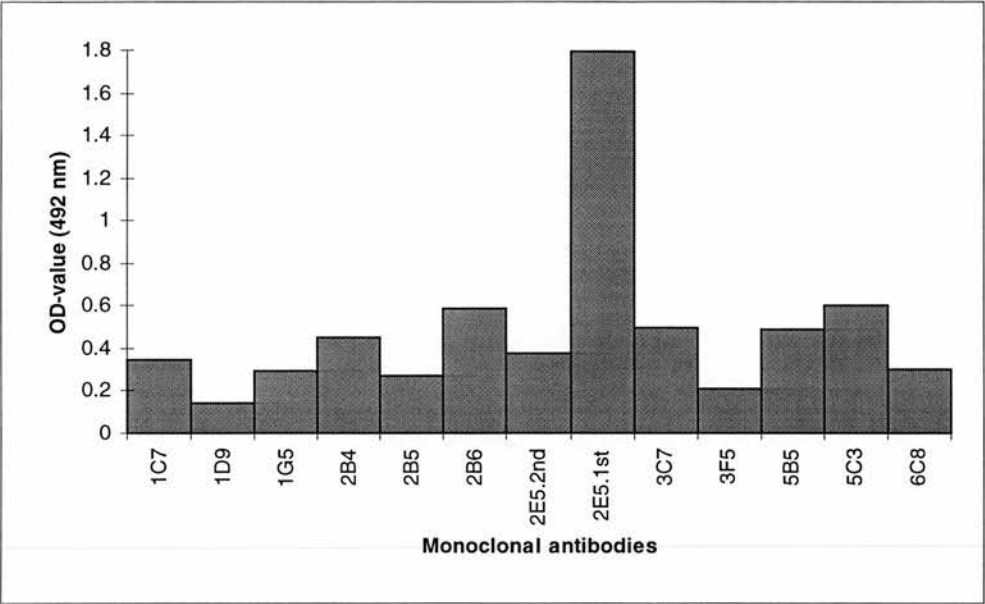
When the mab ascites were scored by IFT on a ++++ to + scale 17 mabs were judged +++, 4 +++ and 6+. The IFT scoring did not appear to relate directly to the ELISA OD values nor was there any apparent relationship between IFT score and mab isotype (Table 3.4).

The ELISA OD values of the ascites (Table 3.4) were compared statistically with the ELISA OD values from the third limiting dilution of the mabs (Table 3.1). Both columns produced skewed curves (left). To restore normality both columns were logged. The correlation was found to be $r = 0.538$. Using Fisher's "tanh"

Table 3:4 Ranking of the 27 monoclonal antibodies based on their ELISA OD value of the ascitic fluid diluted 1:200

	Mab designation	Isotype	ELISA OD value	IFT score
1	6E8	G2a	1.99	++++
2	2E5 (first fusion)	G1	1.79	+++
3	9C3	G2b	1.40	++++
4	13C10	G1	1.31	++++
5	8D7	G2b	1.23	++++
6	10E6	G3	1.02	++++
7	8B6	G1	0.70	++++
8	7C9	G1	0.68	++++
9	6E2	G2b	0.61	+
10	5C3	G1	0.60	++++
11	2B6	M	0.59	++++
12	8G5	G1	0.57	++++
13	13E4	G1	0.52	++++
14	3C7	G2a	0.50	+
15	5B5	G2b	0.49	+++
16	11B6	G1	0.47	++++
17	2B4	G1	0.45	++++
18	11D7	A	0.39	++++
19	2E5 (second fusion)	G2a	0.38	+
20	11E4	G1	0.38	+
21	1C7	G1	0.35	+
22	6E11	G2a	0.35	+
23	6C8	G2b	0.30	++++
24	1G5	G1	0.29	++++
25	2B5	G1	0.27	++++
26	3F5	A	0.21	+++
27	1D9	A	0.15	+++

Figure 3.2: Diagram of the corrected optical density (OD) values obtained from each mab ascites, in an indirect ELISA at 1:200 dilution. All the mabs were examined in one test. Results of the first 13 mabs are illustrated in the top diagram and the other 14 mabs in the diagram below.



transformation (Beyer, 1968), the value was found to have a p-value of 0.002. Therefore there was a very strong correlation between the supernatant OD value of the third limiting dilution and the OD of the ascitic fluid diluted 1/200.

3.2.6 TITRATION OF MAB ASCITES

In order to test the reactivity of the mab ascites at higher dilutions, they were tested at log₁₀ dilutions in an indirect ELISA. Taking ≥ 0.2 OD as the lowest positive end point only five mabs (5B5, 6E8, 9C3, 10E6, 13C10) were still reactive at 1:1,000,000. Twelve mabs (1G5, 2B6 2E5 (1st fusion), 5C3, 6E2, 6E11, 8B6, 8D7, 8G5, 11B6, 11D7, 13E4) were positive at a 1;100,000 dilution, and seven mabs (1C7, 1D9, 2B4, 2E5 (2nd fusion), 3C7, 7C9, 11E4) were positive at a 1:10,000 dilution. All the mabs were positive at a dilution of 1:1000. (Table 3.5).

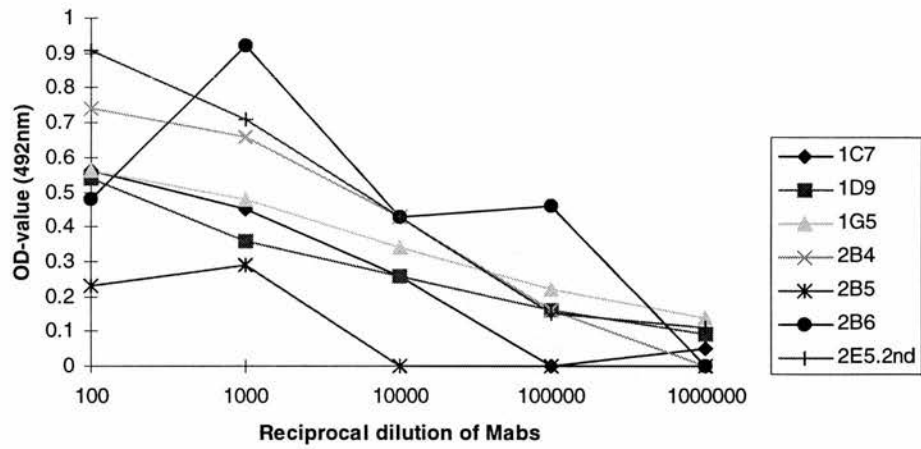
Titration curves of the ELISA OD values of the mab ascites are shown in Figure 3.3a to 3.3d. The majority of the mab ascites had maximum binding affinity at 1:100 dilution, but 2B5, 2B6, 6E11, 7C9, 9C3, 10E6 and 13C10 had the same or higher ODs at 1:1000. Only 1 mab (3C7) had a maximum OD at 1:10,000 dilution although the majority of the mabs bound effectively at this dilution. Of the 5 high affinity mabs which gave an OD value > 0.2 at a dilution of 10^{-6} , 4 were later (Chapter 4) shown to be reacting with a 40kDa protein and only 1 (5B5) was against a 65kDa protein.

Table 3:5 Titration of Mabs (Ascites) against orf virus in an indirect ELISA

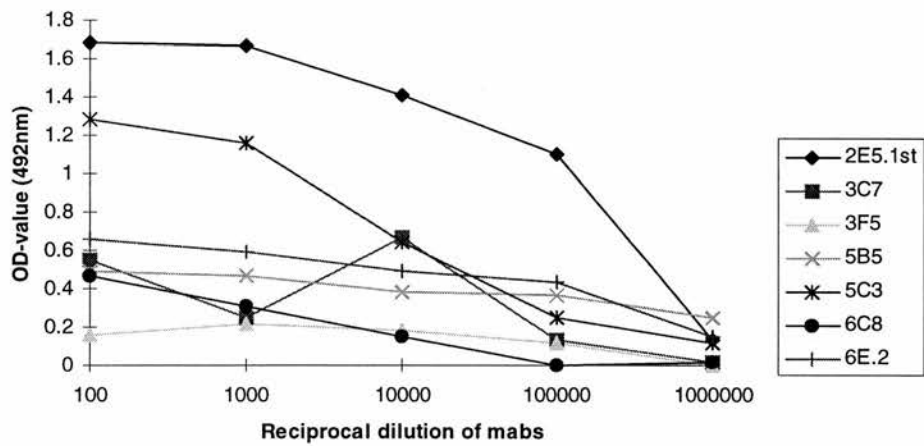
	Mab designation	OD value at 10⁻² dilution	OD value at 10⁻³ dilution	OD value at 10⁻⁴ dilution	OD value at 10⁻⁵ dilution	OD value at 10⁻⁶ dilution
1.	1C7	0.56	0.45	0.26	0.00	0.05
2.	1D9	0.54	0.36	0.26	0.16	0.09
3.	1G5	0.56	0.48	0.34	0.22	0.14
4.	2B4	0.74	0.66	0.43	0.16	0.00
5.	2B5	0.23	0.29	0.00	0.00	0.00
6.	2B6	0.48	0.92	0.43	0.46	0.00
7.	2E5(second fusion)	0.91	0.71	0.43	0.15	0.10
8.	2E5 (first fusion)	1.68	1.67	1.41	1.10	0.13
9.	3C7	0.55	0.25	0.67	0.13	0.01
10.	3F5	0.16	0.22	0.18	0.12	0.00
11.	5B5	0.49	0.47	0.38	0.37	0.25
12.	5C3	1.28	1.16	0.64	0.25	0.11
13.	6C8	0.47	0.31	0.15	0.00	0.02
14.	6E2	0.66	0.59	0.49	0.43	0.15
15.	6E8	2.46	2.30	2.27	1.80	0.43
16.	6E11	0.51	0.76	0.17	0.31	0.04
17.	7C9	0.75	0.79	0.60	0.15	0.09
18.	8B6	0.88	0.65	0.44	0.36	0.16
19.	8D7	1.94	1.57	1.00	0.37	0.12
20.	8G5	0.68	0.32	0.24	0.24	0.00
21.	9C3	2.47	2.47	2.04	1.16	0.30
22.	10E6	1.63	1.65	1.39	0.66	0.22
23.	11B6	0.94	0.73	0.58	0.36	0.15
24.	11D7	0.61	0.53	0.38	0.27	0.14
25.	11E4	0.48	0.41	0.24	0.06	0.03
26.	13C10	2.45	2.45	2.03	0.84	0.20
27.	13E4	0.74	0.66	0.50	0.28	0.13

Figure 3.3: Binding affinity curves of 27 monoclonal antibodies grouped into A, B, C and D. The mabs were serially diluted and reacted with NP40 ELISA antigen of orf-11 virus. The mabs are shown numerically. Groups A, B, C contain 7 mabs and group D has 6 mabs only. Please note that the OD value scales differ from figure to figure to accommodate the variable reactivities of the different mabs.

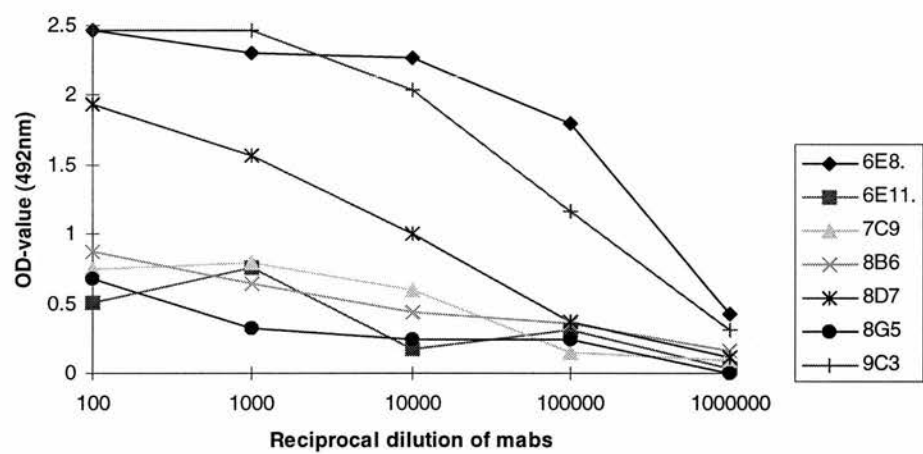
A: Binding affinity of monoclonal antibodies



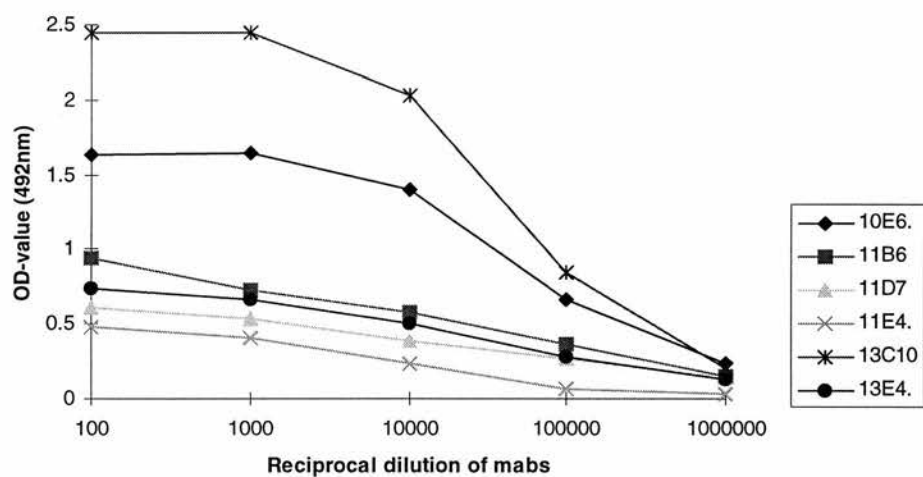
B: Binding affinity of monoclonal antibodies



C: Binding affinity of monoclonal antibodies



D: Binding affinity of monoclonal antibodies



3.3 DISCUSSION

Two immunisation regimes were used. Purified orf virions and a lysate of orf virus infected cell cultures were both successful in inducing mouse lymphocytes that secreted anti-orf virus antibodies. From the first fusion using purified virus, 21 hybridomas were produced, 10 of which were cloned and used to produce ascites. In the second fusion 25 out of 41 hybridomas secreting orf virus antibodies were successfully cloned and used to produce ascitic fluid. The success of these two fusions will be added to the previously reported data on mabs produced against poxviruses (Morrison *et al.*, 1985; Kitamoto *et al.*, 1986a; Kitamoto *et al.*, 1987; Oie and Ichihashi, 1987; Czerny and Mahnel, 1990; Kitamoto *et al.*, 1990; Lard *et al.*, 1991; Payne, 1992; Ouchi *et al.*, 1992; Czerny *et al.*, 1994).

Both the screening systems, immunofluorescence test (IFT) and the enzyme linked immunosorbent assay (ELISA), were sensitive and able to detect specific hybridomas that secreted anti-orf virus immunoglobulin. The two tests have been widely used for screening hybridomas during mab production (Collins *et al.*, 1984; Kitamoto *et al.*, 1986a; Comitti *et al.*, 1987; Czerny and Mahnel, 1990; Kitamoto *et al.*, 1990; Lard, 1991; Ouchi *et al.*, 1992; Czerny *et al.*, 1994b).

The ELISA optical densities recorded for hybridoma supernatant fluids during the cloning stage, showed differences from hybridoma to hybridoma. Some hybridomas had high OD on first cultivation and then dropped considerably thereafter (1C7, 2B5, 3C7, 3F5, 5B5). That decrease in the OD could be attributed first, to gradual elimination of cells which were producing the highest antibodies. Secondly, it is possible that hybridoma cells at the beginning of the fusion were more active in immunoglobulin secretion, but were less stable, with the stable but low secreting hybridoma line being cloned. That behaviour was not generalised for all the hybridomas though, since the OD value of others like 2B4 and 6E8 remained around the same value at each stage of the cloning process.

The value of the Mab isotyping is to confirm whether or not monoclonal antibodies have been sufficiently cloned so that only a single isotype is detected. All the 27 hybridomas each produced a single isotype, providing evidence that they had been effectively cloned.

The class or sub-class of the isotype is determined, by the structure of the heavy chain constant regions (Tizard, 1987; Roitt, 1988; Harlow and Lane, 1988). No correlation was seen between the strength of the OD value in the ELISA and the IgG1, IgG2a and IgG2b isotypes. No particular association of mab IgGs with ELISA OD value emerged, with the 13 IgG1, 4 IgG2a and 4 IgG2b mabs having a range of high and low ODs. The three IgA mabs, however, had low OD values. The OD levels with the IgA mabs were significantly reduced; mab supernatant ($P=0.03$) and ascitic fluid ($P=0.01$). Lard *et al.*, (1991) also used two different immunisation protocols, group (1) with killed orf virus and group (2) with lysate of live virus antigen. Mabs produced from the first group were predominantly IgG3 and IgG1, and only around one tenth of them were IgM, whereas the majority of the group (2) mabs were IgM but the exact numbers were not given. It was concluded that vaccination regimes may possibly have an influence on the type of isotype obtained. Unfortunately, because they didn't report any ELISA OD values for their mabs it was not possible to compare them with our mabs.

It was observed that some mice when injected with hybridoma cells had a tendency to produce a solid tumour rather than ascitic fluid. Nine of the 135 Balb/c mice used developed solid tumours. Three had been injected with 5B5, two with 8D7, one with 5C3 one with 6E8, one with 10E6 and one with 11B6. There was no obvious reason for this, because other mice inoculated with the same hybridomas produced ascitic fluid. It is possible that the hybridoma cells had been injected into tissue of an internal organ rather than the peritoneal cavity and that could have lead to the development of solid tumours (Entrican, G., personal communication). On the other hand, Hoogenraad and colleagues (1983), are of the opinion, that some hybridoma

cell lines predominantly produce solid tumours, because of instability of the hybridomas as a result of prolonged propagation.

When the ELISA OD values of all the 27 hybridoma culture supernatants at the third limiting dilution (final) and of the OD values of their homologous mabs (ascitic fluid) at 1:100 or 1:1000 concentration were compared the values were comparable confirming the stability of the cloned hybridomas and the suitability of the ascitic fluids for characterisation of the mabs as described in the next chapter.

CHAPTER 4
CHARACTERISATION OF MONOCLONAL ANTIBODIES

4.1 INTRODUCTION

This chapter describes the characterisation of the mabs whose production was reported in Chapter 3.

Immunoblotting and radio-immunoprecipitation are the methods commonly used to determine the reactivity of mabs against denatured proteins and thereby determine the molecular weight of the protein with which they react (Goldstein *et al.*, 1982; Collins *et al.*, 1984; Van den Hurk *et al.*, 1984; Rodriguez *et al.*, 1985; Kitamoto *et al.*, 1986a; Payne, 1992; Czerny *et al.*, 1997).

In addition, mabs have also been examined for their ability to neutralise viral infectivity. Both neutralising and non-neutralising mabs have been produced, for example, against bovine herpes virus type-1 (Collins *et al.*, 1984; Okazaki *et al.*, 1986), vaccinia virus (Rodriguez *et al.*, 1985; Czerny and Mahnel, 1990) and orf virus (Lard *et al.*, 1991; Czerny *et al.*, 1997). A mixture of two mabs to vesicular stomatitis virus has been shown to provide better and more rapid neutralisation than when each mab was used alone (Volk *et al.*, 1982).

There are few reports of neutralising mabs against poxviruses. Monoclonal antibodies that neutralise vaccinia virus prevent the virus uncoating (Rodriguez *et al.*, 1985). The uncoating inhibition was determined by measuring the level of conversion of vaccinia virus particles to subviral cores using velocity sedimentation in sucrose gradients.

Lard *et al.*, (1991) were unsuccessful in neutralising orf virus with a pool of mabs but a recent report has described a mab capable of neutralising orf virus (Czerny *et al.*, 1997).

This chapter describes characterisation of the mabs which have been produced and the identification of the molecular weight of orf virus proteins they recognise.

4.2 RESULTS

4.2.1 WESTERN BLOTTING

Two different orf virus preparation were used to determine mab reactivity:- purified orf virus and a lysate of orf - 11 infected cells. Using purified orf virus only 5 mabs (2E5 1st fusion, 6E8, 6E11, 8D7, 10E6) showed any reactivity, and each of these recognised a protein of 40kDa (Fig 4.1). In a confirmatory test using purified antigen both ascitic fluid and hybridoma culture supernatant of the same five mabs detected the 40kDa protein (Fig 4.2). The supernatant containing the mab 6E11 only weakly detected the 40kDa protein, indicative of a low immunoglobulin content. Because the strips were left for one minute longer in the DAB substrate to develop further, some non-specific bands developed.

Attempts were made to increase the number of mabs reacting in western blotting by omitting 2ME and heat in the preparation of the antigen. Western blotting was done using purified antigen without 2ME in loading buffer but boiled for 5 minutes, and with 2ME but unboiled. No new reactions were noted but the five mabs reported above again detected the 40kDa protein in denatured and non-denatured conditions.

When the mabs were tested against a lysate of orf-11 infected cells the same five mabs again detected a 40kDa protein. In addition, three mabs (3F5, 7C9, 11D7) recognised a protein of molecular weight 65kDa (Fig 4.3). The other 19 mabs did not react.

The sheep anti-orf virus hyperimmune serum, designated 1847 reacted with five polypeptides in the purified orf virus preparation with apparent molecular weights of 200kDa, 40, 29, 25 and 22kDa (Fig 4.1). When orf virus infected cell lysate was used as antigen six polypeptides were visualised with molecular weights of 200, 65, 40, 29, 25 and 22kDa (Fig 4.3). Prominent recognition of the 65kDa in the cell lysate by the hyperimmune serum indicated its importance as an immunodominant protein.

Figure 4.1: Western blot with purified orf-11 virus. Lane. 1 - Molecular weight markers; Lanes 2 and 10 - hyperimmune anti-orf virus serum; Lanes 3 and 9 negative mab ascites (VPM20). Lane 4 -2E5 (1st fusion); Lane 5 - 6E8; Lane 6 - 6E11; Lane 7 - 8D7 and Lane 8 - 10E6.

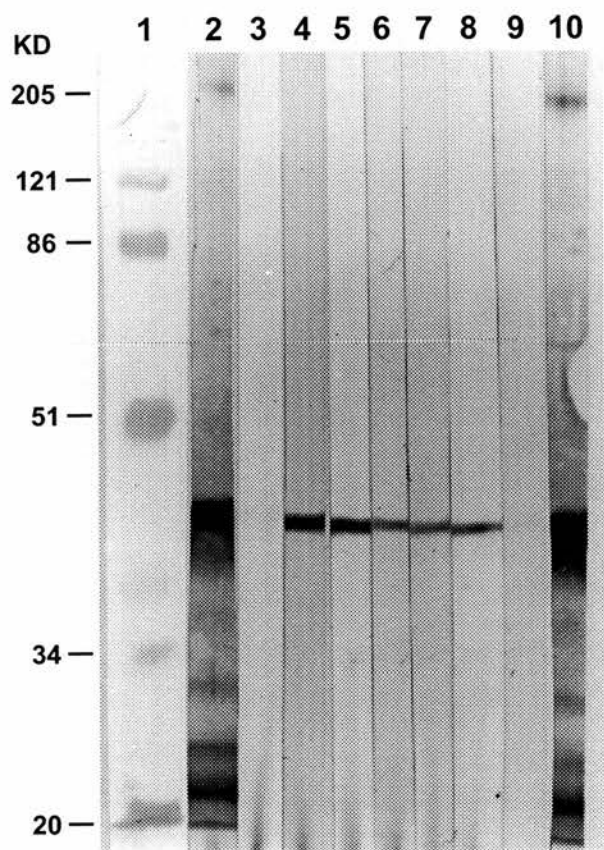


Figure 4.2: Western Blotting using purified orf-11 virus with either mab culture supernatants (lanes 2 to 6) or ascites (lanes 10 to 14). Lanes 1 and 9 negative sheep serum (260). Lanes 2 and 10 - 2E5 (1st fusion); lanes 3 and 11 - 6E8; lanes 4 and 12 - 6E11; lanes 5 and 13 - 8D7; lanes 6 and 14 - 10 E6; lanes 7 and 15 - negative ascites (VPM20); lanes 8 and 16 - hyperimmune sheep anti-orf virus serum (1847).

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

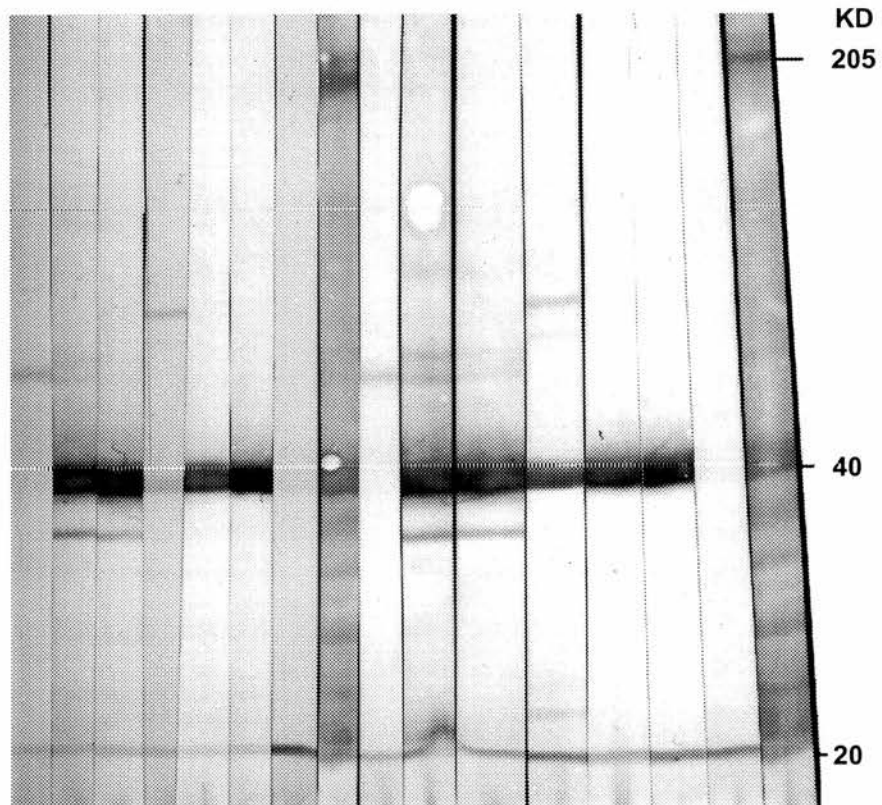
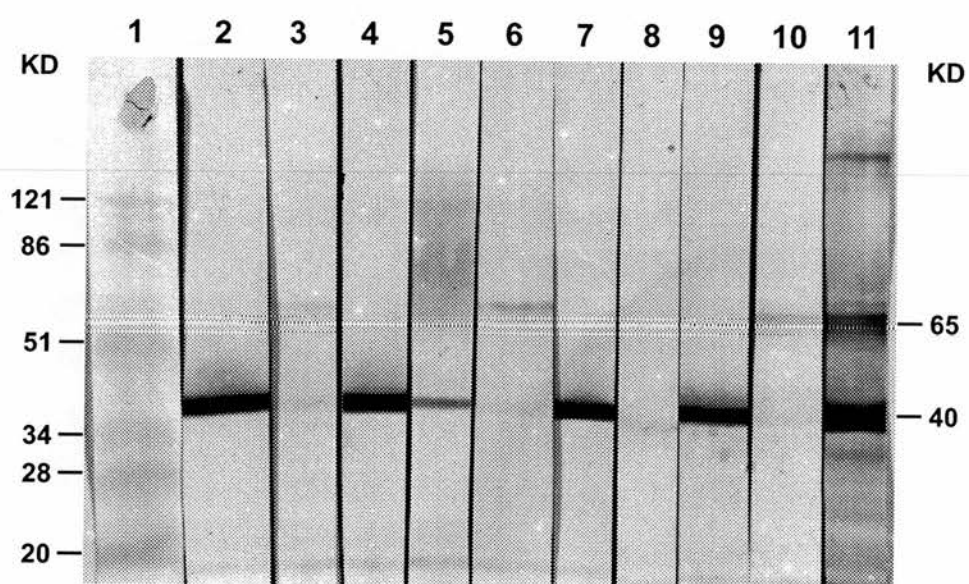


Figure 4.3: Western blot using orf-11 cell lysate antigen. Lane 1 - molecular weight markers; Lane 2 - 2E5 (1st fusion); Lane 3 - 3F5; Lane 4- 6E8; Lane 5 - 6E11; Lane 6 - 7C9; Lane 7 - 8D7; Lane 8 - negative mab (VPM20); Lane 9 - 10E6; Lane 10 - 11D7; Lane 11 - hyperimmune sheep anti-orf virus serum (1847).



4.2.2 RADIOIMMUNOPRECIPITATION

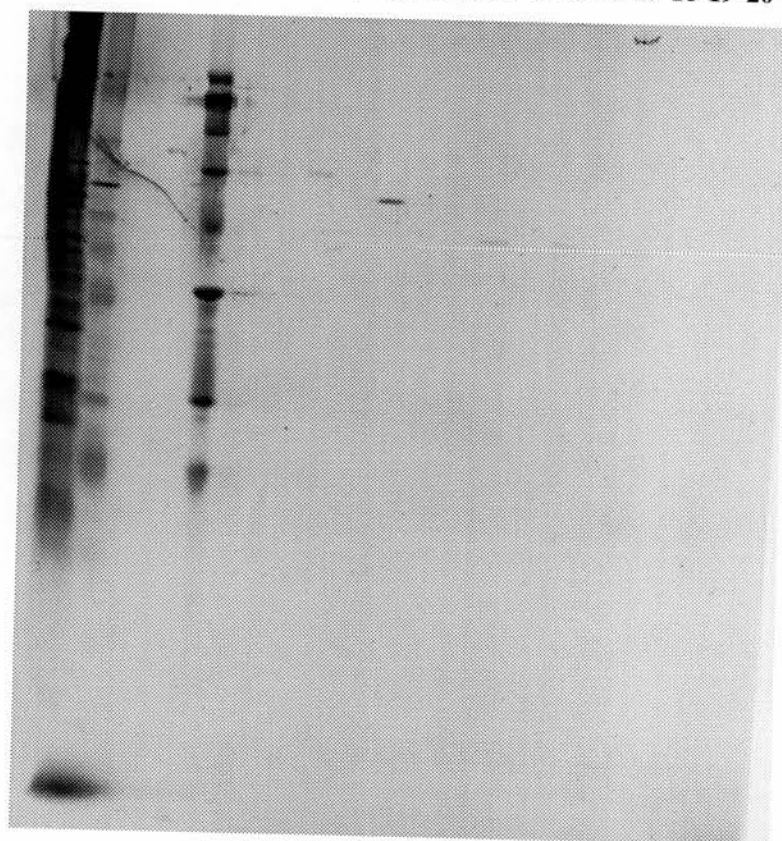
Radioimmunoprecipitation with mabs was used to detect antigen specificity in those mabs which did not react with denatured antigens. Twenty-lane gels were used to test all the mabs at least twice. Representative gels are shown. Control lanes were included in each gel and gave consistent results. Controls included lysates of:- uninfected unprecipitated cells, orf-infected unprecipitated cells, uninfected cells mixed with hyperimmune serum, infected cells mixed with hyperimmune serum, along with rainbow markers. Results of one gel (Figure 4.4) show five mabs detecting 3 different proteins of orf-11 virus. The mab 6E2 detected a 65kDa protein (lane 8), the mab 6E11 reacted with a 50kDa protein (lane 10) and three other mabs 8D7, 9C3, and 10E6 recognise a 40kDa protein in lanes 13, 15, and 17.

The second gel (Figure 4.5) also shows five mabs that produced bands. As before 8D7 mab reacted with a 40kDa protein (lane 8). A 50kDa protein, was detected by mab (6E11) (lane 10) and mabs 5B5, 1G5 produced bands with a 65kDa molecular weight protein in lanes 11 and 14. The 13C10 mab immunoprecipitated a 40kDa protein (lane 20).

The third gel shows films developed after one week (Figure 4.6a) and after 6 weeks (Figure 4.6b). After one week single bands were visible in lanes 1, 2, 3, 6, 10 and 17. These were predominantly 40kDa bands in lanes 2, 6, 10 and 17. While 3 of these lanes contained mabs it was noteworthy that only the 40kDa protein was visible in lane 10, that contained the hyperimmune serum. Other mabs detected a protein of 65kDa protein in lane 1 and a 50kDa protein in lane 3. After six weeks of film development (Figure 4.6b) all the bands seen previously were more easily observed. Four other weak 65kDa bands detected by 8B6, 8G5, 11B6 and 11E4 in lanes 5, 14, 18 and 20 became apparent and another mab 9C3 produced a 40kDa band in lane 15. Other orf virus specific bands were detected by the hyperimmune serum in lane 10 and similar bands were also apparent in some of the

Figure 4.4: Radioimmunoprecipitation of orf virus antigens with mab ascites. Lane 1-uninfected unprecipitated cells; Lane 2-orf infected unprecipitated cells; Lane 3 - uninfected cells with hyperimmune sheep serum 1847; Lane 4 - uninfected cells mixed with another hyperimmune serum 267; Lane 5- Rainbow markers; Lane 6 - infected cells with hyperimmune sheep serum 1847; Lane 8 - 6E2; Lane 10 - 6E11; Lane 13 - 8D7; Lane 15 - 9C3; Lane 17 - 10E6. Other lanes contained mab ascites that showed no immunoprecipitation of any orf virus protein.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

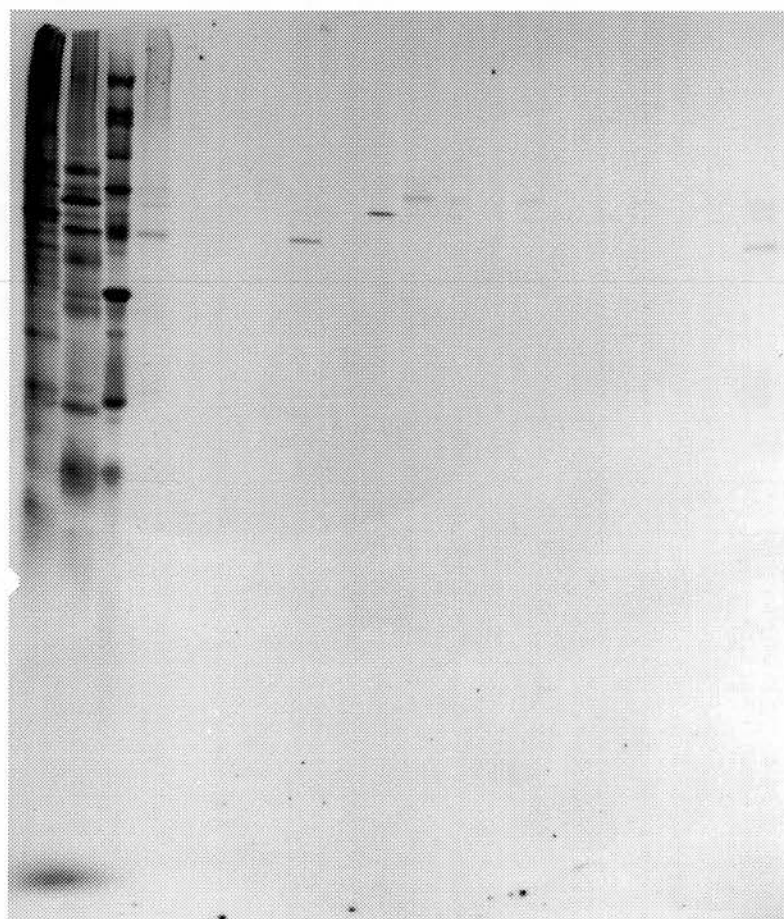


kd

▲ 65
▲ 50
▲ 40

Figure 4.5: Radioimmunoprecipitation of orf virus antigens with mab ascites. Lane 1-uninfected unprecipitated cells; Lane 2 - orf infected unprecipitated cells; Lane 3 - Rainbow markers; Lane 4 - orf virus infected cells with hyperimmune serum; Lane 5 - ; Lane 6 - ; Lane 7 - ; Lane 8 - 8D7; Lane 10 - 6E11; Lane 11 - 5B5; Lane 14 - 1G5; Lane 20 - 13C10. Other lanes contained mab ascites that showed no immunoprecipitation of any orf virus protein.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20



kd

▲ 65
▲ 50
▲ 40

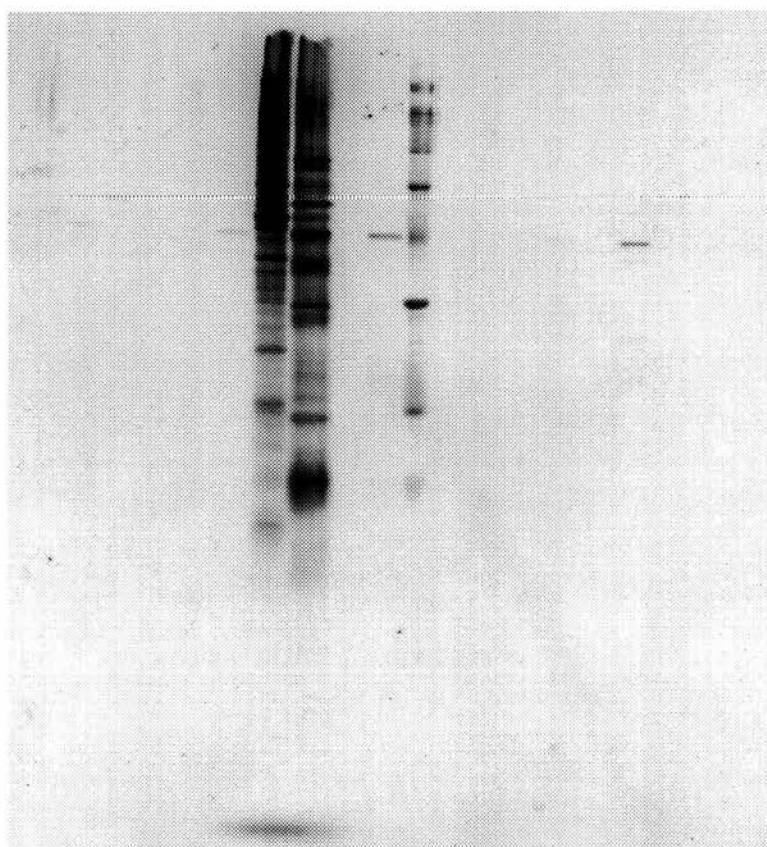
Figure 4.6 (a + b): Radioimmunoprecipitation of orf virus proteins (a) film after one week exposure and (b) after 6 weeks exposure. Lane 1 - 6E2; Lane 2 - 6E8; Lane 3 - 6E11; Lane 4 - 7C8; Lane 5 - 8B6; Lane 6 - 8D7; Lane 7 uninfected unprecipitated cells; Lane 8 - orf virus infected precipitated cells; Lane 9 - uninfected cells with hyperimmune serum; Lane 10 - orf infected cells with hyperimmune serum; Lane 11 - Rainbow markers; Lane 12 - negative serum 243; Lane 13 - VPM20; Lane 14 - 8G5; Lane 15 - 9C3; Lane 16 - 9D5; Lane 17 - 10E6; Lane 18 - 11B6; Lane 19-11D7; Lane 20 - 11E4.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

kd

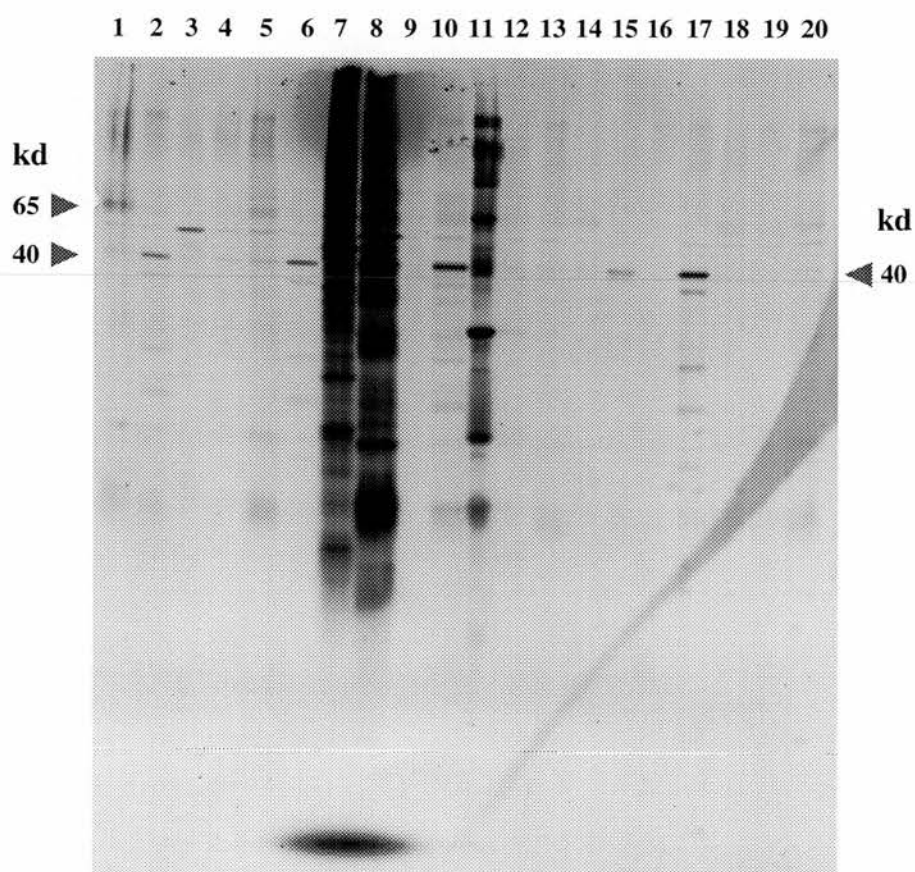
65 ▶

40 ▶



kd

◀ 40



other lanes, suggesting that despite the washing with RIPA buffer some residual orf virus proteins were present.

In total, 21 mabs produced bands by RIPA, thirteen mabs immunoprecipitated a 65kDa protein, seven were against a 40kDa protein and one was against a 50kDa protein (Table 4.4). Four of the mabs reacting with 40kDa protein by Western blotting were found to detect a protein of the same molecular weight in RIPA while the fifth (6E11) reacted with a 50kDa protein. Interestingly the three mabs (3F5, 7C9, 11D7) which had been shown to bind to the 65kDa protein in Western blotting did not apparently immunoprecipitate proteins using RIPA.

4.2.3 PULSE-CHASE EXPERIMENTS

The time of production of the 40kDa and 65kDa proteins was studied using sequential harvesting of infected cells every three hours. Results gathered after one week of the film being exposed, revealed that the 40kDa was first precipitated and detected 18 hrs post - infection (pi) and continued to appear in increasing amounts 21, 24 and 27 hrs pi (Figure 4.7), but when the film was developed after six weeks the protein was detectable as early as 15 hrs pi onwards up to 27 hrs pi. The intensity of the 40kDa bands in lanes 9 and 10 appeared increased over the bands in lanes 7 and 8, suggesting that increasing quantities of the 40kDa protein were produced at 24 and 27 hrs p i.

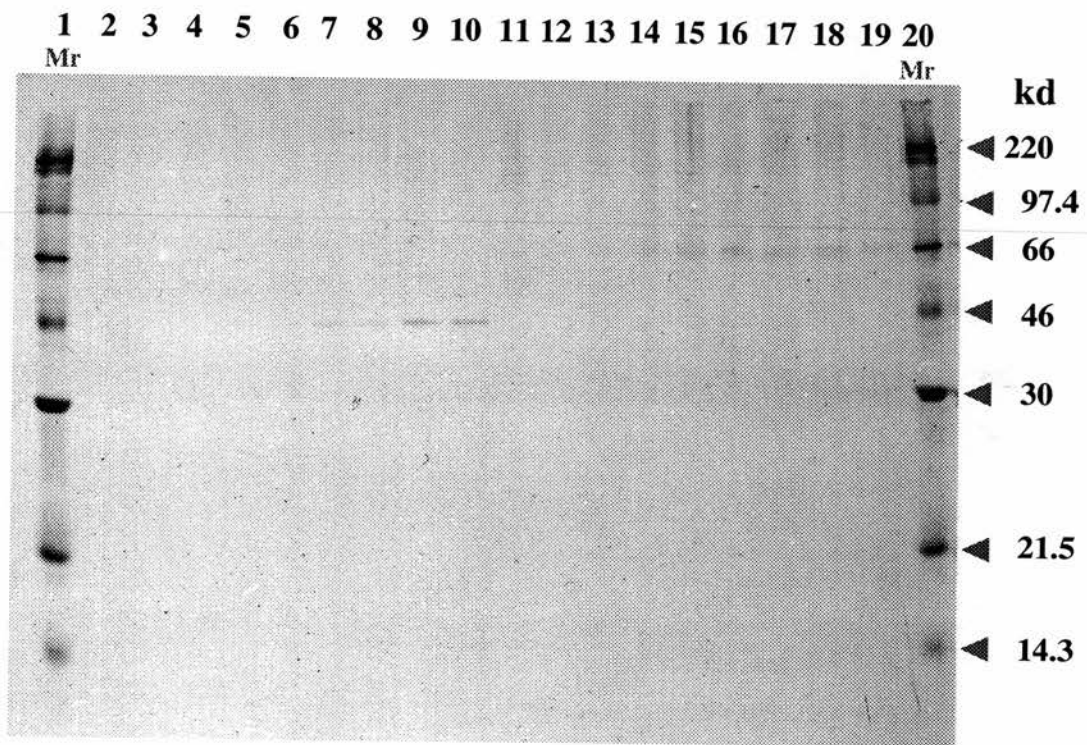
The 65kDa band was first detected at 12 hrs pi and an apparent increase in protein immunoprecipitated occurred up to 27 hrs. This was observed in the films developed after one week and six weeks exposure. There was a slight increase in the strength of the bands after six weeks development.

4.2.4 SERUM NEUTRALISATION TEST (SNT)

Neutralisation tests with each of the 27 ascites against 100 TCID₅₀ of orf-11 virus showed that none of the mabs had neutralising ability, based on the appearance of viral cpe recorded on the third day after infection. At this time the positive control hyperimmune serum 1847 neutralised virus in all wells between neat and 1/8 serum

dilutions and in one of the two wells at 1/16 dilution such that no viral cpe was detectable in any of these wells. At higher dilutions of serum viral cpe had killed at least 50% of the cells. The end point neutralisation titre of the hyperimmune serum was recorded as 1/16. When the plates containing the mab dilutions were examined all the wells had viral cpe which had killed at least 50% of the cells. There was no difference in appearance of the cpe in relation to the dilution of the mabs or between the mabs. It was concluded that none of the mabs showed any evidence of neutralisation even when used as undiluted ascites.

Figure 4.7: Precipitation of the 40kDa and 65kDa proteins in a pulse-chase experiment. Lanes 1 and 20 - Rainbow markers; Lane 2 - 2E5 (1st fusion) and orf virus antigen 3 hours after harvesting; Lane 3 - 2E5 and orf virus antigen 6 hours after harvesting; Lane 4 - 2E5 after 9 hours; Lane 5 - 2E5 after 12 hours; Lane 6 - 2E5 after 15 hours; Lane 7 - 2E5 after 18 hours; Lane 8 - 2E5 after 21 hours; Lane 9 - 2E5 after 24 hours; Lane 10 - 2E5 after 27 hours; Lane 11 - 6E2 3 hours after harvesting; Lane 12 - 6E2 after 6 hours; Lane 13 - 6E2 after 9 hours; Lane 14 - 6E2 after 12 hours. Lanes 15 to 19 - 6E2 after 15 to 27 hrs at 3 hourly intervals as above.



4.2.5 STATISTICAL ANALYSIS OF THE ODs OF THE ANTI-40 kDa AND ANTI-65 kDa MABS

For comparing the ODs of the hybridoma supernatants which recognised the 40 kDa protein to the hybridoma supernatants which recognised the 65 kDa protein (Table 4.1a), the OD values were first log-transformed to approximate a normal distribution. The mean logged ODs of the 40 kDa group was 0.142 and the mean logged ODs of the 65 kDa group was -0.292. Using a t-test, the t value was 4.26. At 22 degrees of freedom there was a highly significant difference (P-value =0.0013) between the ODs of the mabs which recognise the 40 kDa and the 65 kDa proteins.

Exactly the same approach was used for data of ascites ODs (Table 4.1b). The mean of the logged anti-40 kDa mabs was 0.029 and for the 65 kDa group was -0.350. The t value was 3.94. Once again a highly significant difference (P-value =0.0034) between the ODs of the 40 kDa and the 65kDa proteins was obtained.

4.2.6 SCREENING OF THE MABS AGAINST 18 VVOV-RECOMBINANTS BY IFT

Supernatant from each cloned hybridoma culture was tested against all eighteen recombinants. A recombinant vaccinia virus with a β -galactosidase (β gal) insert was used as a control along with cover slips of orf infected and non-infected cells. In the screening of the mabs against 18 VVOV-recombinants 17 mabs recognised the recombinants 79, 285, and 286 and only 4 mabs 2E5 (1st fusion), 6E8, 8D7, 10E6 reacted against the two overlapping recombinants 245 and 247. The other 6 mabs did not react with any of the 18 recombinants (Table 4.2).

None of the mabs reacted against negative control β gal Lister recombinant and none detected the Lister strain of vaccinia virus.

The IFT profile of the mabs against the VVOV-recombinants showed that the panel of orf mabs react with at least two different orf virus proteins which can be mapped to the virus genome and ascribed a putative function.

Table 4.1.a Relationship of ELISA OD mab hybridoma cell cupernatants (third limiting dilution) with the protein reactivity detected by Western blot and/or RIP.

40 kDa (ODs)	65 kDa (ODs)	Unknown kDa Mab (ODs)
2.48	1.22	0.94
2.47	0.93	0.61
2.45	0.88	0.57
2.02	0.85	
1.13	0.59	
0.79	0.51	
0.76	0.49	
0.67	0.48	
	0.46	
	0.44	
	0.44	
	0.42	
	0.37	
	0.36	
	0.35	
	0.20	
n = 8	n=16	n=3

Table 4.1.b Relationship of ELISA OD of mab ascites at 1:200 dilution with the reaction detected by Western blot and/or RIP.

40 kDa (ODs)	65 kDa (ODs)	Unknown kDa mab (ODs)
1.99	0.70	0.38
1.79	0.68	0.27
1.40	0.61	0.15
1.31	0.59	
1.23	0.57	
1.02	0.52	
0.60	0.50	
0.35	0.49	
	0.47	
	0.45	
	0.39	
	0.38	
	0.35	
	0.30	
	0.29	
	0.21	
n = 8	n=16	n=3

Table 4.2: Reactivity of 27 mabs with CV-1 cells infected with 18 VVOV recombinants plus VV with a β gal insert using an indirect immunofluorescence test. Reactivity with the recombinants is shown as positive or negative and strength of reactivity with orf 11 virus is recorded as a scale of + (weak) to ++++ (strong) fluorescence.

		VVOV RECOMBINANTS																β gal	Orf
		79	80	82	85	86	96	97	212	213	215	216	243	245	247	283	285	286	330
MABS	1C7	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-
	1D9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	1G5	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-
	2B4	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-
	2B5	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-
	2B6	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-
	2E5	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-
	2E5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	3C7	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-
	3F5	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-
	5B5	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-
	5C3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	6C8	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-
	6E2	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-
	6E8	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-
	6E11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	7C9	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-
	8B6	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-
	8D7	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-
	8G5	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-
	9C3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	10E6	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-
	11B6	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-
	11D7	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-
	11E4	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-
	13C10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	13E4	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-
Totals	27	17	-	-	-	-	-	-	-	-	-	-	-	4	4	-	17	17	-

4.2.7 REACTIVITY OF THE MABS AGAINST TWO NEW VVOV - RECOMBINANTS

Two new recombinants (380) and (436) became available toward the end of this research. VVOV-recombinant 380, contains an orf virus genome insert of the overlapping region between the 245 and 247 VVOV recombinants. The recombinant 380 was sequenced and was found to contain more than one gene. Thereafter the orf virus genome insert in 380, was cut and a further VVOV-recombinant, known as 436, expressing a single orf virus gene (FIL) was made. The 380 and 436 recombinants were produced as reported by Roberts (1996) and kindly supplied by Dr A Mercer. All 8 mabs which had shown any evidence of reacting with a 40kDa protein were tested for their activity against the 2 new VVOV recombinants by IFT. Only three mabs 2E5(1st fusion), 8D7, 10E6 reacted with both of the recombinants 380 and 436 confirming their reactivity with the product of the orf virus gene FIL.

One of the four mabs (6E8) which originally reacted strongly with overlapping recombinants 245/247 did not react with the two new recombinants 380 and 436 (Table 4.3).

Table 4.3: Reactivity of four of the mabs reacting with the 40kDa protein with CV-1 cells infected with 380 and 436 recombinants.

Mabs	Recombinants VVOV- 245/247	Recombinant VVOV-380	Recombinant VVOV-436	Orf-11
2E5 (MRI)	+	+	+	+
6E8	+	-	-	+
8D7	+	+	+	+
10E6	+	+	+	+

4.2.8 SUMMARY OF CHARACTERISTICS OF THE 27 MABS

In order to allow easy reference to the main features of the 27 mabs their principal characteristics have been summarised (Table 4.4).

Table 4.4: Summary of principal characteristics of 27 monoclonal antibodies

Mab number characterisation	Isotype	OD ELISA values	Western blot	RIPA	VVOV
1C7	G1	0.35	-	65kDa	79,285, 286
1D9	A	0.14	-	-	-
1G5	G1	0.29	-	65kDa	79,285, 286
2B4	G1	0.45	-	65kDa	79,285, 286
2B5	G1	0.27	-	-	79,285, 286
2B6	M	0.59	-	65kDa	79,285, 286
2E5 (2 nd fusion)	G2a	0.38	-	-	-
2E5 (1 st fusion)	G1	1.79	40kDa	40kDa	245, 247, 436
3C7	G2a	0.50	-	65kDa	79,285, 286
3F5	A	0.21	65kDa	-	79,285, 286
5B5	G2b	0.49	-	65kDa	79,285, 286
5C3	G1	0.60	-	40kDa	-
6C8	G2b	0.30	-	65kDa	79,285, 286
6E2	G2b	0.61	-	65kDa	79,285, 286
6E8	G2a	1.99	40kDa	40kDa	79,245, 247
6E11 (1 st fusion)	G2a	0.35	40kDa	50kDa	-
7C9	G1	0.68	65kDa	-	79,285, 286
8B6	G1	0.70	-	65kDa	79,285, 286
8D7	G2b	1.23	40kDa	40kDa	245, 247, 436
8G5	G1	0.57	-	65kDa	79,285, 286
9C3	G2b	1.40	-	40kDa	-
10E6	G3	1.02	40kDa	40kDa	245, 247, 436
11B6	G1	0.47	-	65kDa	79,285, 286
11D7	A	0.39	65kDa	-	79,285, 286
11E4	G1	0.38	-	65kDa	79,285, 286
13C10	G1	1.31	-	40kDa	-
13E4	G1	0.52	-	65kDa	79,285, 286

4.3 DISCUSSION

4.3.1 WESTERN BLOTTING

Western blotting provides a specific method of identifying the molecular weight of a particular region of the antigen with which an antibody reacts. Cumulative information from Western blots using orf-11 antigen showed that out of 27 mabs only five reacted and all these detected a 40kDa protein. When Western blotting was done using a lysate antigen of orf-11 the same five mabs again reacted with a 40kDa protein, and three other mabs detected a 65kDa protein. The limited number of proteins recognised by the mabs is in accordance with a previous study of two orf virus mabs, one of which appeared to react with a 38-40kDa protein and the other with a 40-43kDa protein (Lard *et al.*, 1991). When Czerny *et al.*, (1997) described three mabs against orf virus (strain D1701) 2 different proteins of molecular weight 39 and 22 kDa were recognised by western blotting.

Failure of the other 20 mabs to produce a detectable reaction to antigens in Western blotting is likely to be because they are against conformational epitopes which have been disrupted by the presence of reducing agents (Harlow and Lane, 1988).

It was not the main goal to study serum from immunised sheep in Western blots. Nevertheless, Western blot with hyperimmune anti-orf virus serum (1847) was used as a positive control, and six polypeptides of orf-11 virus were detected with approximate molecular weights of 200, 65, 40, 29, 25 and 22kDa. This is in agreement with the number of proteins previously reported. (Chand *et al.*, 1994; Chin and Petersen, 1995). However, the size of the bands differed in these reports, Chand and colleagues found 67, 42, 32, 26, and 18kDa proteins, whereas, 82-84, 43, 38, 36, 25 and 23kDa proteins were described by Chin and Peterson (1995).

Generally, up to 35 orf virus proteins can be resolved in SDS PAGE, but only 6 seem to induce a sufficiently strong immune response to be detected by Western blotting (Balassu and Robinson, 1987; McKeever *et al.*, 1987).

4.3.2 RADIOIMMUNOPRECIPITATION

The advantage of using immunoprecipitation for antigen detection is that it is flexible and extremely sensitive. Twenty-one out of the 27 mabs precipitated proteins of orf-11 virus. These data contribute to the previous mab precipitation of poxviruses reported for vaccinia virus (Panye, 1992); cowpoxvirus (Kitamoto *et al.*, 1987); swine poxvirus (Ouchi *et al.*, 1992) and orf virus (Schindler, 1995).

The 6E11 mab reacting against the 40kDa proetin in western blotting consistently precipitated a 50kDa protein in RIPA. The reason for this is unclear, as all the other mabs which recognised the 40 kDa protein in western blots precipitated a 40kDa protein in RIPA. Clearly the presence of protease inhibitors (Aprotonin and phenylmethyl sulfonyl fluoride (PMSF) during the cell lysis have lessened, if not completely prevented protein proteolysis as appeared with the other 40kDa mabs (2E5 MRI, 6E8, 8D7, 10E6) detecting the same 40kDa in RIPA. Therefore, one may speculate that 40 and 50kDa may be due to behaviour of that (6E11) mab. In studies of pestivirus (bovine virus diarrhoea and border disease viruses) it has been noticed that mabs which react with P120 of non-cytopathic virus, precipitate two proteins P54 and P80 from the cytopathic strain of virus (Dutia *et al.*, 1990). The two proteins P54 and P80 of the cytopathic form are homologous and equal to P120 (NCP form) and both proteins P54 and P80 have identical antigenic determinants to that of P120. Similarly, one group of mabs produced against Bovine Herpesvirus type 1 (BHV-1) were found to precipitate antigens with two different molecular weights, GVP3 (180K) and GVP9 (91K) (Van den Hurk *et al.*, 1984). This indicates that the mabs may have been reacting with an antigenic determinant in one polypeptide that is a part of a complex with one or two other polypeptides in the cell lysate.

Results of the precipitation showed that 13 mabs were reacting with a 65kDa protein, 1 mab with a 50kDa protein and 7 mabs with the 40kDa protein. From 5 fusions, Schindler (1995) produced 75 mabs, precipitating eight different proteins of orf virus. Interestingly the majority (33) were against a 66kDa protein and 18 were against a 41kDa protein. Other proteins detected had molecular weights of 40kDa (10 mabs), 39kDa (5), 42kDa (3), 100kDa (2), 21kDa (2) and 14kDa (2).

Three (3F5, 7C9, 11D7) of the six mabs that did not react in radio-immunoprecipitation were previously found to detect a 65kDa protein in western blotting a phenomenon which has also been described by (Goldstein *et al.*, 1982; Goding, 1986). Bearing in mind that different mabs have different behaviour, failure of the mabs mentioned above to precipitate the 65kDa antigen, could be attributed to: firstly, the immunocomplexes were of low avidity and the three washings with RIP buffer may have affected the stability of the precipitation; secondly, that some antigenic determinants of the viral antigen recognised by these mabs were denatured during the solubilisation stages.

In a recent report on immunoprecipitation of orf virus (NZ2 strain) with hyperimmune serum, eight bands with molecular weights of 108, 85, 72, 69, 47, 43, 28 and 27kDa were successfully precipitated (Mercer *et al.*, 1997).

4.3.3 PULSE CHASE EXPERIMENT

In order to establish the time when the 65kDa and 40kDa protein could first be detected pulse chase experiments were conducted and results have shown the 65kDa could be found from 12hrs post-infection to 27hr, which coincides with results with swinepox virus (SPV) reported by (Ouchi *et al.*, 1992). The 40kDa was first detected at 15hrs and was detectable up to 27hrs pi.

A similar piece of work to this study was done by Ouchi *et al.*, (1992). They generated mabs against swine poxvirus (SPV), and one of their mabs (SP14) cross-reacted and precipitated a 65kDa polypeptide from a lysate of orf virus infected cells and rabbit fibroma virus as well as from a lysate of the SPV infected cells. It is possible, but it may not be necessary that mab SP14 and some other mabs raised against orf-11, for instance (1C7) are recognising a common epitope in orf-11, and the orf virus designated as I wate BT-9, Rabbit fibroma (OA) and the three swine pox viruses (OV-1, PP-1, Kianagawa). This, however, requires substantiation. The 65kDa was found to be a late protein, based on the absence of a precipitating band, when cytosine-1B-D-arabinofuranosyl Hcl (Ara C; 50µg/ml) was added to the cells at the time of infection. The Ara C prevents late genes from being expressed, a method

which has also been used to determine early and late proteins of vaccinia, cowpox and Shope fibroma viruses (Ikuta *et al.*, 1979; Ouchi *et al.*, 1992).

The other approach for determining early and late orf virus genes is through sequencing information. So far all the orf virus genes published appear to align with equivalent vaccinia virus genes. Interestingly, all appear to occur in the same order and orientation on their respective genomes, despite the G+C content difference between the parapoxviruses and the orthopoxviruses (Naase *et al.*, 1991; Fleming *et al.*, 1992; Sullivan *et al.*, 1994; Mercer *et al.*, 1995).

The gene expressing the 40 kDa protein has been sequenced and has been found to be a late protein (Roberts, 1996). Further information about this protein, which was detected from 15 hours post-infection in this study, will be described later. It is, however, concluded from the data presented that both the 65 and 40kDa proteins recognised by mabs are late orf virus proteins.

4.3.4 NEUTRALISATION TEST

Because mabs may be generated against epitopes within polypeptides, it is not unexpected to produce mabs that have no neutralising activity of their homologous virus. All the 27 mabs produced did not neutralise orf-11 virus, and this is in agreement with previous reports (Lard *et al.*, 1991; Czerny *et al.*, 1994b, 1997). There is only mab (4D9) which has been described to have neutralising capability against orf virus, and that mab reacted with a 39 kDa orf envelope protein (Czerny *et al.*, 1997).

Monoclonal antibodies that bind to the 14kDa envelope protein of vaccinia virus are the mabs which were found to neutralise vaccinia virus infectivity (Rodriguez *et al.*, 1985; Chang *et al.*, 1995). Three mabs detected the 14kDa of VV which is believed to be a conserved protein in orthopoxviruses (Rodriguez *et al.*, 1985).

Lack of the neutralisation ability of the 27 mabs against orf-11, could be related to the high challenge dose (100 TCID₅₀) of virus employed, or the involvement of

multiple antigenic determinants in neutralisation which the mab did not bind to. The virus was, therefore, still able to attach, penetrate and multiply in the cells.

Envelope proteins (structured proteins) have always been considered important, because of their role in neutralising antibody production. Vaccinia virus appears to have two forms of envelope, extracellular enveloped virus (EEV) and intracellular mature virus (IMV) (Payne, 1979; Payne, 1980; Vanderplasschen and Smith, 1997).

Recently the two forms (EEV and IMV) were closely studied (Vanderplasschen and Smith, 1997). Interestingly, the efficiencies of their binding to a variety of cell lines was different and difference in their binding to the surface of cells treated with digestible enzyme was also noted.

The EEV was found to have up to 10 associated proteins that did not resolve in the IMV (Payne, 1979). Remarkably, the EEV and IMV seem to have different binding cell receptors based on the results of a mab against IMV, which bound to the cell surface and inhibited IMV binding but not EEV (Vanderplasschen and Smith, 1997).

From the point of variation noted within vaccinia EEV and IMV this may support the earlier report (Mercer *et al.*, 1994) that vaccinia virus and orf virus don't cross-protect against each other, since each of them has distinct envelope proteins and are of course distinct viruses of two genera.

4.3.5 REACTIVITY WITH VVOV - RECOMBINANTS

Over the last 14 years the genetic engineering of poxviruses has provided a very significant tool for researchers to study the biology of poxviruses, the expression of important exotic genes in recombinant poxviruses and their use as vectors in recombinant vaccines (Moss, 1996; Paoletti, 1996). Certainly the availability of the VVOV-recombinants (Mercer *et al.*, 1997) was helpful in the initial characterisation of the mabs. Mabs recognising recombinants either reacted with the three (79, 285, 286) all of which encode a 14Kb region in the Kpn1 A fragment in the middle of the genome, or reacted with two recombinants (245/247) which overlap by

approximately 10Kb. Six mabs (1D9, 2E5 (orf-11), 5C3, 6E11, 9C3, 13C10) showed no reactivity with any of the recombinants all of which were reactive with sheep hyperimmune serum in an IFT and produced variable fluorescent intensities (Mercer *et al.*, 1997).

Together the results of western blotting, RIP and IFT showed that the 79, 285 and 286 recombinants are expressing the 65kDa protein and the two overlapping recombinants 245/247 are expressing the 40kDa protein. These results are in agreement with others who concluded that these two proteins were immunodominant (McKeever *et al.*, 1987; Lard *et al.*, 1991; Chand *et al.*, 1994; Czerny *et al.*, 1994b; Schindler, 1995; Azwai *et al.*, 1995; Chin and Petersen, 1995; Roberts, 1996).

Inability of six mabs to detect any of the recombinants suggest that these mabs were raised against proteins not expressed by the recombinants or the recombinant machinery did not produce the protein in a form recognised by the mabs.

Further studies aimed to check reactivity of all the eight mabs to the 40kDa protein against the two latest recombinants (380) and (436). Surprisingly, only the 3 mabs, 2E5 1st fusion, 8D7, 10E6, were reactive with gene products of both recombinants. Five of the eight mabs detected identical bands in western blots, seven of the eight reacted with the 40kDa protein in RIP and four gave positive reactions with the original recombinants 245/247. These results evidently suggest the mabs are reacting against the same polypeptide. With the five mabs that showed no reactivity with recombinants 380 and 436 it is likely these mabs are against different epitopes of the 40kDa polypeptide *in vivo* which are not being expressed by these two recombinants. The possibility that there is another protein of approximately 40 kDa molecular weight cannot be excluded however, especially in the light of the report of mabs against protein with molecular weights of 39, 40, 41 and 42 kDas (Schindler, 1995).

The VVOV-recombinant (436) contains a gene (F1L) which is homologous to the H3L gene of vaccinia virus which encodes an envelope protein (Roberts, 1996). The F1L was found to have a 28.4% amino acid identity to H3L. Although, the overall

level of homology may look low, nonetheless, high similarities were seen between different regions of the two sequences, for instance, 43.1% of identity of (OV) F1L residues 188 to 297 to that of VAC H3L and about 62.5% similarity of the C-termini of the two proteins (F1L residues 319 to 334). Furthermore, the OV F1L region 188 to 203 had 83.1% homology with H3L (VAC). Although the region was short it is believed that the motif may have high significance for the function of these proteins (Roberts, 1996). The H3L gene has formally been characterised and found to encode for a major envelope protein of VV (Zinoviev *et al.*, 1994).

The F1L gene, therefore, codes for the third major structural protein to be identified in orf virus after the 10kDa polypeptide which is homologous to the 14kDa of VV (Naase *et al.*, 1991) and the 42kDa polypeptide which is homologous to the 37kDa protein of VV (Sullivan *et al.*, 1994).

Identification of the 65kDa protein remains elusive. The fact that 16 of the 27 mabs studied were against this protein suggest it is produced in significant quantities in orf-virus infected cells. The genome region encompassed by the 79/285/286 VVOV recombinants has not been fully sequenced but sequencing of this region is being pursued. Preliminary evidence suggests homology to the VV A3L gene which encodes a major core protein (T. Fitzgerald, personal communication).

CHAPTER 5
CROSS-REACTIVITY OF THE MABS AGAINST
PARAPOXVIRUSES AND CAPRIPOXVIRUS

5.1 INTRODUCTION

Viruses belonging to the same genera usually have some antigenic relationship to each other (Woodroffe and Fenner, 1962; Sheshberadaram *et al.*, 1986; Ikuta *et al.*, 1979; Bennett, 1989; Libeau and Lefevre, 1990; Munz *et al.*, 1993).

The significance of antigenic similarity among viruses, has provided the basis for attempts to use a vaccine available against one virus to protect against another member of the same genus. Vaccinia virus has been used to protect against smallpox (Brown *et al.*, 1986; Bennett, 1989). Buttner (1981) successfully managed to vaccinate a group of cattle with an attenuated orf virus vaccine; all the vaccinated cattle were protected against challenge with bovine papular stomatitis. In capripoxvirus infections a single vaccine has been used to protect against both sheeppox and goatpox (Kitching, 1986; Kitching *et al.*, 1986b; Carn, 1993). In morbillivirus infections live attenuated rinderpest vaccine is used to vaccinate sheep and goats to control peste-des-petits-ruminants (PPR) (stated by Libeau *et al.*, 1992).

The other importance of antigenic similarity is that hyperimmune serum to one member can be used for diagnosis of other members in the same genus. Antiserum to vaccinia virus is reported to be useful for detecting cowpoxvirus, rabbit pox, ectromelia as well as homologous antigen (Woodroffe and Fenner, 1962; Ikuta *et al.*, 1979). The three capripoxviruses (lumpy skin, sheeppox and goatpox) are serologically indistinguishable (Carn, 1993). Convalescent antisera from cattle infected with BPS were found to react by IFT with BPSV and cross-react with MNV and OV (Rosenbusch and Reed, 1983).

Monoclonal antibodies are powerful reagents for discriminating between isolates of similar or different type species (Libeau and Lefevre, 1990; Ouchi *et al.*, 1992). Mabs have been raised against different strains of poxviridae, namely vaccinia virus (Rodriguez *et al.*, 1985; Czerny and Mahnel, 1990; Payne, 1992), cowpox virus (Kitamoto *et al.*, 1987; Kitamoto *et al.*, 1990), monkeypoxvirus (Ichichashi and Oie,

1988), swinepox virus (Ouchi *et al.*, 1992) and orf virus (Lard *et al.*, 1991; Schindler, 1995).

The antigenic relationship between parapoxviruses and capripoxviruses is unclear. Early studies by Bennett *et al.*, (1944) reported that goats recovered from goatpox are protected against goatpox and orf virus infections, but orf virus infected goats are susceptible to goatpox virus. This finding would indicate an antigenic relationship between capripoxvirus and parapoxvirus, but other workers have found no protection between the two diseases (Sharma and Dhanda, 1971; Renshaw and Dodd, 1978).

This section presents the use of the mabs to differentiate between parapoxviruses and the results of screening the mabs against capripoxvirus.

5.2 RESULTS

5.2.1 CROSS-REACTIVITY OF THE MABS BY ELISA

Cross-reactivity of the mabs was conducted against eight different parapoxviruses. Four (orf-11, D1701, scabby mouth, NZ2) were from sheep, two from cattle (Bovine papular stomatitis and Milker's node), one from seal (seal ppv) and one from a red squirrel (squirrel ppv). Mab ascites were used at 1/200 dilution. Difficulty was encountered in producing a satisfactory ELISA antigen with the scabbymouth virus such that even the hyperimmune serum (1847) produced an unconvincing OD value. The results with this virus have been included, however, since the two mabs (6E8 and 2E5 1st fusion) producing the highest ODs with orf-11 antigen had ODs >0.2 and 3 other mabs showed evidence of reactivity when ODs greater to the OD of the mean of 8 negative wells plus 3 standard deviations were considered as positive (Table 5.1). Similar uncertainty hung over the seal ppv and squirrel ppv antigens. No specific positive reference sera against these viruses were available. While the hyperimmune anti-orf serum reacted with the squirrel ppv antigen no cross-reactivity was seen with the seal ppv antigen. No mabs showed any evidence of reactivity with the seal or squirrel ppv antigens.

Comparison of the mab reactivity will be restricted to the 5 ppv antigens which gave strongly positive (>0.9) OD values with the hyperimmune serum. When an ELISA OD cut-off value of the mean plus 3 standard deviation of the negative mab was used all the 27 mabs detected orf-11 virus (Table 5.1). For the other sheep parapoxviruses 11 mabs were positive against D17O1, and 22 mabs recognised NZ2. Whereas for the cattle parapoxviruses 13 mabs were binding to MNV and 5 mabs reacted with BPSV.

Cross-reactivity of each of the individual mabs showed differences between their ELISA OD values for the orf virus strains and the other parapoxviruses studied (Table 5.1). Some mabs produced high OD for the orf strains and others were moderate to low. The highest OD values were produced by six of the 40kDa mabs (2E5 (1st fusion), 6E8, 8D7, 9C3, 10E6 and 13C10) which was consistent with the earlier findings in chapter 3. Of the other two anti-40 kDa mabs mab 5C3 demonstrated a moderate OD value with orf-11 virus only, whereas 6E11 with its interesting 40/50 kDa protein binding bound better with BPSV and MNV, suggesting that the reactive polypeptide is more common in cells infected with the bovine parapoxviruses than with the other viruses studied. A similar sort of binding was also produced by 8B6, one of the 65kDa mabs, which produced better binding with BPSV than with any of the other viruses tested. The other 65kDa mabs (1C7, 1G5, 2B4, 2B6, 3C7, 3F5, 5B5, 6C8, 6E2, 7C9, 8G5, 11B6, 11D7, 11E4, 13E4) provided moderate to low OD values mostly with the orf viruses, again reflecting the earlier findings in chapter three.

When the cross-reactivity of the individual anti-40 kDa protein mabs was examined 2E5 (1st fusion) produced higher OD concentration for the orf-11 and NZ2viruses than the D1701 and scabbymouth viruses. Mab 6E8 recorded the highest OD with the three orf viruses, had a high OD against MNV but was negative against BPSV. The mab 8D7, however, had high OD values against the 3 orf and the 2 cattle viruses. The mabs 5C3, 9C3 and 13C10 recorded high ODs with orf-11 virus, compared to the

other viruses. The 10E6 mab produced very similar OD values with all five of the parapoxviruses.

Table 5.1: ELISA OD values of the monoclonal antibodies against different parapoxvirus antigens

	Protein reactivity	Orf-11	D1701	Scabby Mouth	NZ2	Milker's Node	BPS	Seal BJ1880	Squirrel Parapox
MAB									
	kDa								
1C7	65	0.35	0.07	0.0	0.18	0.06	0.14	0	0.01
1D9	-	0.14	0.08	0	0.08	0.02	0.04	0	0
1G5	65	0.29	0.27	0.03	0.20	0.12	0.14	0.02	0
2B4	65	0.45	0.22	0.0	0.24	0.04	0.07	0	0.01
2B5	-	0.27	0.09	0.0	0.02	0.01	0.06	0	0
2B6	65	0.59	0.30	0.0	0.34	0.14	0.01	0.0	0.01
2E5 (2 nd fusion)	-	0.38	0.45	0.07	0.30	0.11	0.05	0.0	0
2E5 (1 st fusion)	40	1.79	0.29	0.25	1.67	0.11	0.02	0	0.01
3C7	65	0.50	0.23	0.03	0.25	0.12	0.01	0.03	0
3F5	65	0.21	0.16	0.06	0.14	0.06	0.28	0.02	0
5B5	65	0.49	0.20	0.04	0.27	0.13	0.03	0	0
5C3	40	0.60	0.19	0	0.0	0	0.0	0	0.01
6C8	65	0.30	0.11	0	0.16	0.03	0.0	0.0	0
6E2	65	0.61	0.27	0.04	0.39	0.18	0	0	0.02
6E8	40	1.99	1.85	0.25	1.88	0.82	0.04	0.0	0
6E11	40/50	0.35	0.30	0.05	0.13	0.99	1.60	0	0.01
7C9	65	0.68	0.38	0.0	0.39	0.03	0.11	0.0	0
8B6	65	0.70	0.32	0	0.51	0.14	0.88	0.0	0.01
8D7	40	1.23	1.35	0.02	0.85	0.63	1.31	0.02	0.01
8G5	65	0.57	0.18	0	0.35	0.09	0.09	0	0
9C3	40	1.40	0.72	0	0.26	0.05	0.14	0.03	0.02
10E6	40	1.02	0.94	0.13	0.88	0.98	1.02	0	0
11B6	65	0.47	0.12	0.03	0.35	0.02	0.03	0.0	0
11D7	65	0.39	0.09	0	0.22	0.03	0.14	0.01	0
11E4	65	0.38	0.10	0	0.25	0.18	0.04	0.0	0
13C10	40	1.31	0.59	0	0.03	0.0	0.0	0.0	0.01
13E4	65	0.52	0.22	0.09	0.36	0.05	0.01	0.01	0
Hyperimmune 1847		1.40	1.40	0.06	1.65	0.93	1.72	0.005	0.34
SPF		0.0	0.04	0.0	0.0	0.01	0.02	0	0.05
VPM20 mean		0.03	0.13	0.01	0.07	0.02	0.06	0.03	0.04
Standard deviation (SD)		0.014	0.054	0.016	0.024	0.028	0.037	0.007	0.052
VPM20 mean + 3 SDs		0.07	0.29	0.06	0.14	0.10	0.17	0.05	0.19

Considering the ELISA results as a whole, four mabs (6E11, 8B6, 8D7, 10E6) were positive against the orf virus strains, MNV and BPSV. Nine mabs (1G5, 2B6, 2E5 (1st fusion), 2E5 (2nd fusion), 3C7, 5B5, 6E2, 6E8, 11E4) reacted with the orf strains and MNV only. Only one monoclonal (3F5) recognized BPSV and not MNV. Ten of the mabs (1C7, 2B4, 6C8, 7C9, 8G5, 9C3, 11B6, 11D7, 13C10, 13E4) were only positive with orf strains and three mabs (1D9, 2B5, 5C3) were reacting only with orf-11 virus antigen.

A statistical analysis of the OD values of the five parapoxviruses was conducted. The OD values were strongly skewed, and a normalising log transformation was successful only for the orf-11 and D1701 variables. In the examination of skewed data, the use of the standard correlation coefficient is inappropriate. Therefore, the non-parametric Spearman rank correlation test was used. The following matrix of results was produced:-

Table 5.2: Spearman rank correlation coefficients of mab ELISA OD values for the five parapoxviruses

	Orf-11	D1701	NZ2	Milker's Node
D1701	0.788			
NZ ₂	0.732	0.644		
Milker's Node	0.387	0.567	0.657	
BPS	0.019	0.263	0.216	0.422

Table 5.3: The P values associated with the above table

	Orf-11	D1701	NZ2	Milker's Node
D1701	≤ 0.002			
NZ ₂	≤ 0.002	≤ 0.002		
Milker's Node	0.018	≤ 0.002	≤ 0.002	
BPS	0.477	0.075	0.160	0.022

Interpretation of the P - values shows that:- All the orf virus strains, are correlated with everything, except with BPS. Milker's node virus is significantly correlated with all the other viruses, while BPS is not significantly correlated with anything except with Milker's node virus.

5.2.2 CROSS-REACTIVITY OF THE MABS BY IFT

All 8 parapoxviruses grew well in FLM coverslip cultures and the IFT with the hyperimmune anti-orf serum gave clear results with all the viruses. The IFT results with the mabs were also clear and showed that eleven mabs recognised all the sheep and cattle viruses but not the seal or squirrel viruses (Table 5.4). Three (8D7, 8G5, 10E6) reacted with each of the sheep and cattle viruses and the seal virus and six (2E5 (2nd fusionI), 2E5 (1st fusion), 3C7, 6E8, 11E4, 13E4) recognised at least 3 of the orf virus strains and MNV, but did not recognise BPSV. Two (5C3, 13C10) reacted only with orf viruses and one mab (9C3) recognised orf virus strains and BPSV only. The 6E11 mab reacted with each of the sheep and cattle PPVS and also the squirrel PPV. One mab (1C7) reacted with all eight PPVS suggesting it is recognising a conserved epitope on the 65kDa antigen. Reactivity of the mabs with the four orf viruses demonstrated that the scabby mouth had fewer recognised epitopes than the other three viruses. All the mabs reacted with orf-11 and twenty-six mabs were positive with D1701 and with NZ2 strain. Only six mabs (1C7, 7C9, 8D7, 8G5, 10E6, 11B6) produced fluorescent reaction with seal ppv and two (1C7, 6E11) with squirrel ppv.

The positive IFT reactions of the mabs against squirrel PPV, revealed granular fluorescence in the cytoplasm of infected cells. The hyperimmune (1847) serum produced a similar fluorescence to that produced by the mabs 1C7 and 6E11 (Figure 5.1).

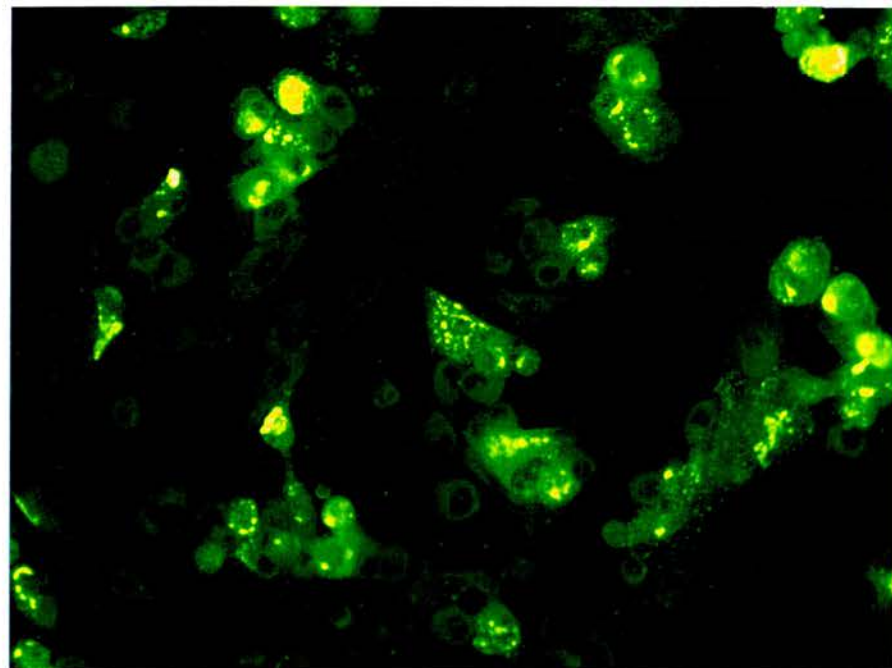
Figure 5.2 (a, b, c, d,) shows the pattern of fluorescence in the cytoplasm of FLM cells infected with the German strain D1701 and screened with four different mabs. Mabs 1G5 and 11E4 produced inclusions (a and c) while the mabs 8D7 and 5C3 produced predominantly a diffuse fluorescence (b and d).

Table 5.4: Cross-reactivity of the monoclonal antibodies against parapoxvirus by IFT.

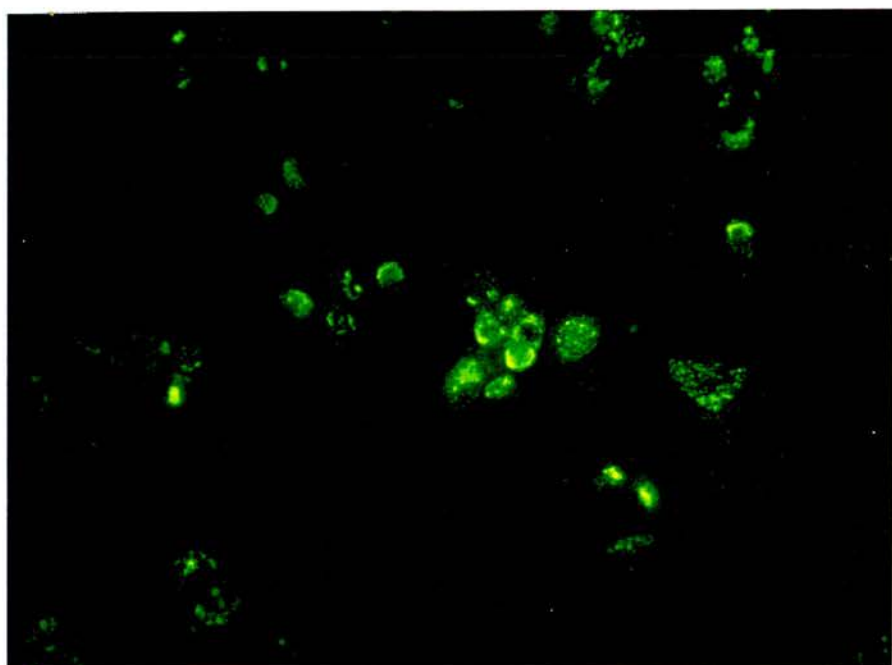
	Protein reactivity kDa	Orf-II	D1701	Scabby Mouth	NZ2	Milker's Node	Bps	Seal BJ1880	Squirrel Parapox
MAB									
	Kda								
1C7	65	+	+	+	+	+	+	+	+
1D9	-	+	-	-	+	+	+	-	-
1G5	65	+	+	+	+	+	+	-	-
2B4	65	+	+	+	+	+	+	-	-
2B5	-	+	+	-	+	+	+	-	-
2B6	65	+	+	-	+	+	+	-	-
2E5 (2 nd fusion)	-	+	+	+	+	+	-	-	-
2E5 (1 st fusion)	40	+	+	+	+	+	-	-	-
3C7	65	+	+	-	+	+	-	-	-
3F5	65	+	+	+	+	+	+	-	-
5B5	65	+	+	+	+	+	+	-	-
5C3	40	+	+	-	-	-	-	-	-
6C8	65	+	+	+	+	+	+	-	-
6E2	65	+	+	-	+	+	+	-	-
6E8	40	+	+	+	+	+	-	-	-
6E11	40/50	+	+	+	+	+	+	-	+
7C9	65	+	+	-	+	+	+	+	-
8B6	65	+	+	+	+	+	+	-	-
8D7	40	+	+	+	+	+	+	+	-
8G5	65	+	+	+	+	+	+	+	-
9C3	40	+	+	-	+	-	+	-	-
10E6	40	+	+	+	+	+	+	+	-
11B6	65	+	+	+	+	-	+	+	-
11D7	65	+	+	+	+	+	+	-	-
11E4	65	+	+	-	+	+	-	-	-
13C10	40	+	+	+	+	-	-	-	-
13E4	65	+	+	+	+	+	-	-	-
Hyperimmune 1847		+	+	+	+	+	+	+	+

Figure 5.1: Colour photographs of immunofluorescent staining of FLM cells infected with squirrel ppv and tested, (a) - with hyperimmune serum (1847), (b) - with the mab 1C7 and (c) - with the mab 6E11. All produced granular fluorescence. Magnification x200.

A



B



C

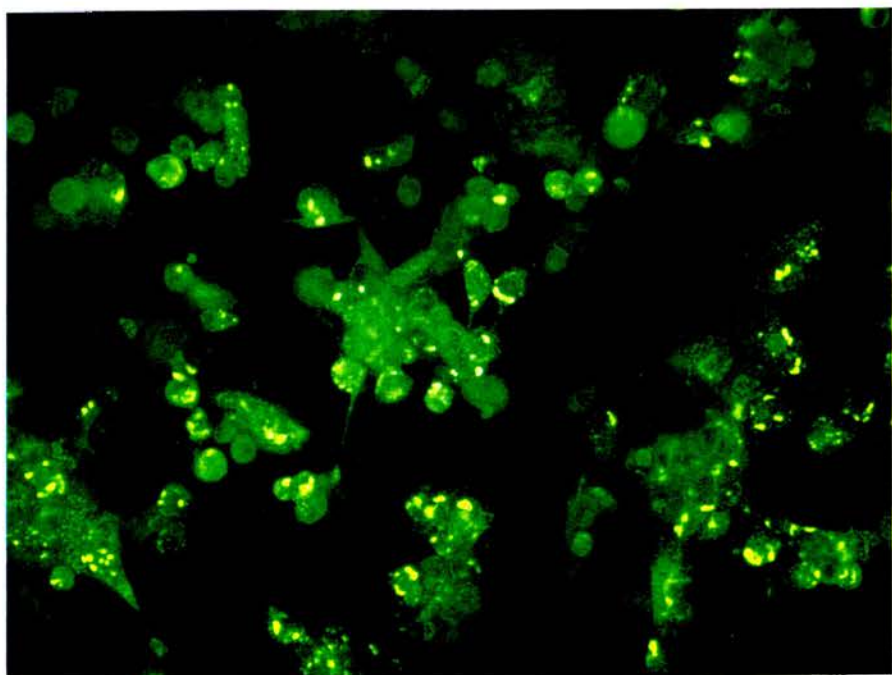
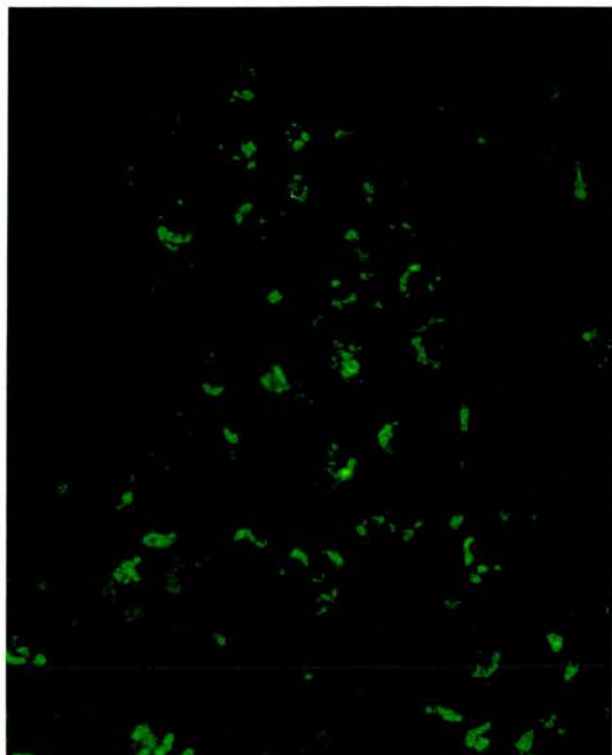
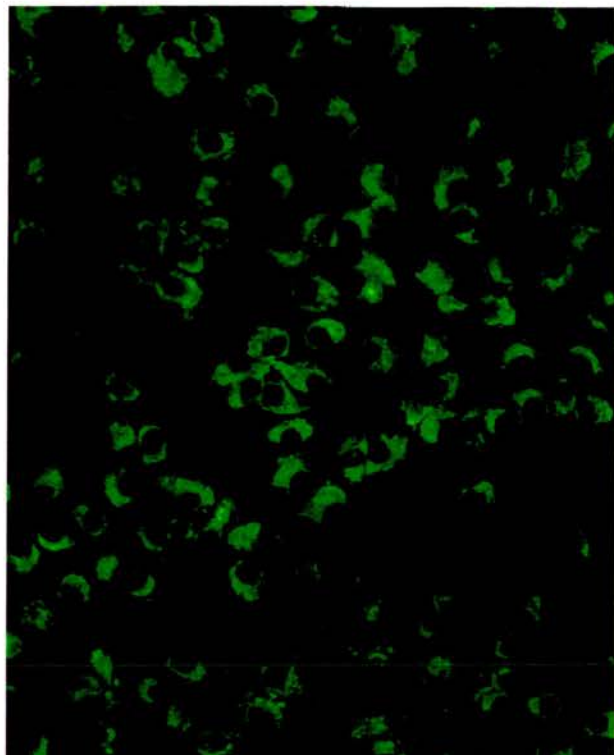


Figure 5.2: Colour photographs of immunostaining patterns of FLM cells infected with the German orf virus (D1701) and detected with four different mabs (a) 1G5 produced inclusions; (b) 8D7 produced diffuse staining; (c) 11E4 also produced inclusions and (d) 5C3 produced diffuse staining. Magnification x 200.

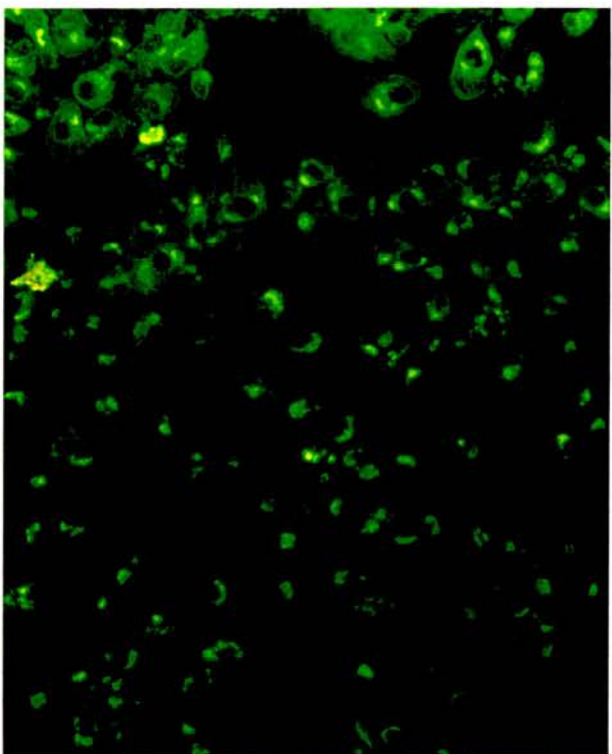
A



B



C



D

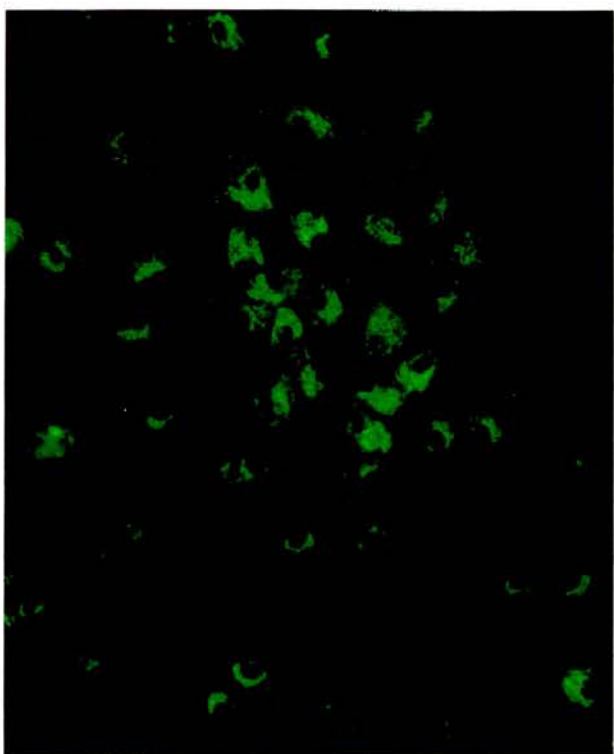
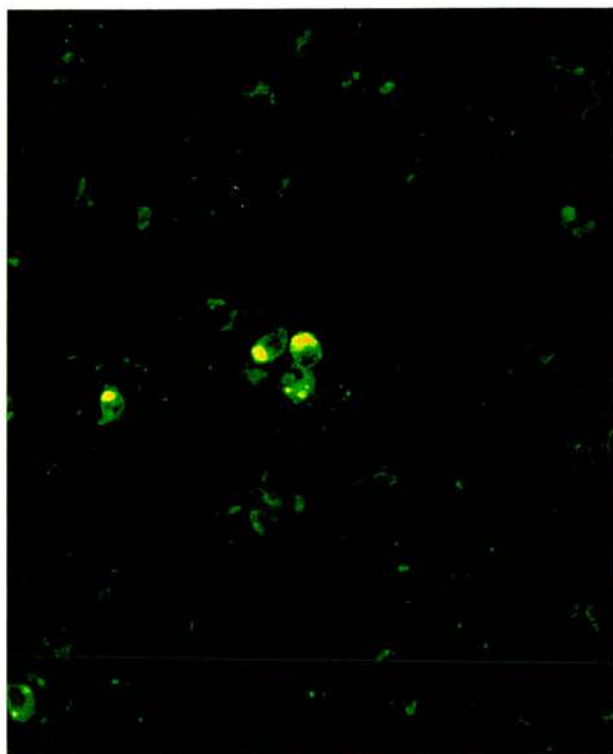
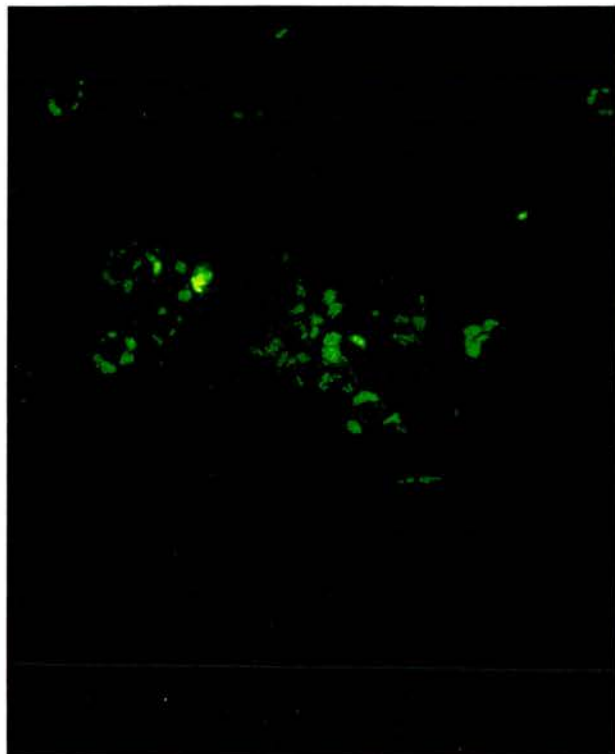


Figure 5.3: Colour photographs of reactivity of four different parapoxviruses by IFT that all showed evidence of both diffuse and inclusion body-type staining, (a) shows fluorescence of seal ppv with the mab 10E6; (b) illustrates fluorescence of BPSV detected by mab 5B5; (c) the mab 6E8 was reacted with MNV, and in (d) the reaction of 5B5 against NZ2 orf virus is shown. Magnification x 200.

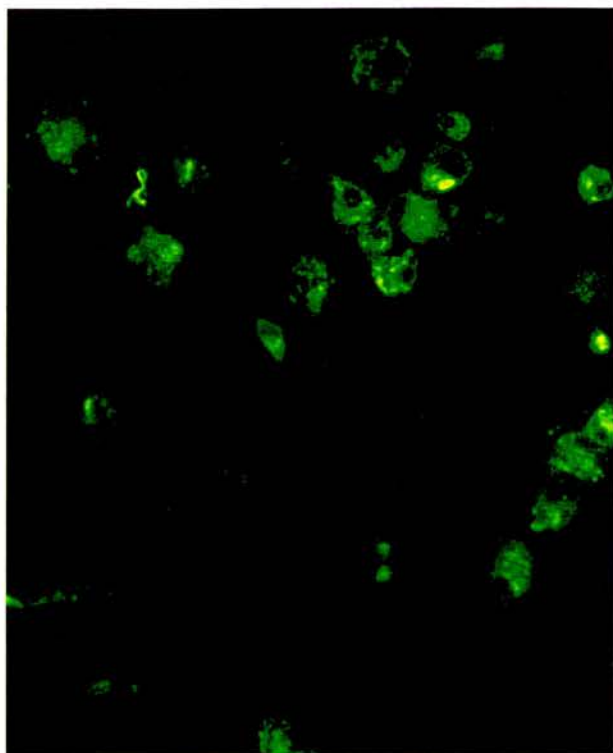
A



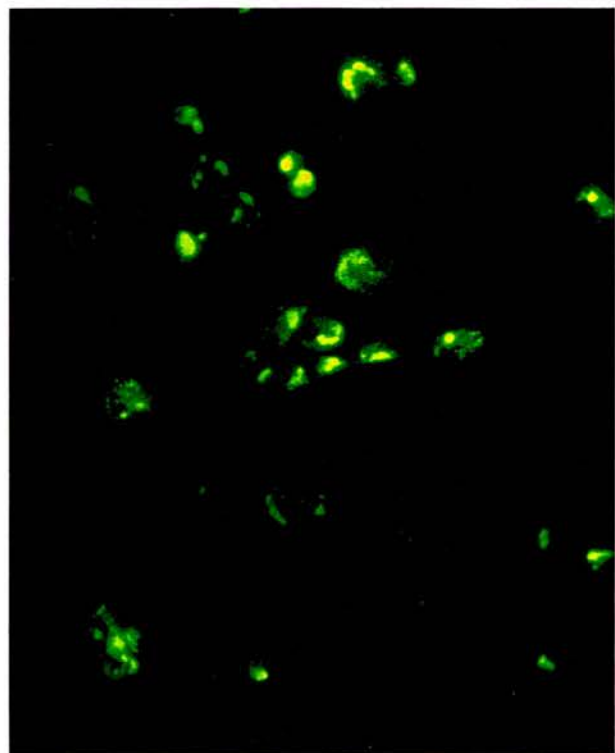
B



C



D



Some examples of fluorescence in which both diffuse and inclusion staining were seen are shown in Figure 5.3 (a, b, c, d).

From figures shown and other reactivities it was noted that most of the 40kDa mabs produced diffuse fluorescence while the 65kDa mabs produced fluorescence characterised by inclusions. However both the 40/50kDa mab and the 65kDa mab produced granular fluorescence with squirrel ppv and in some cases both the diffuse and inclusion types of fluorescence were seen (Figure 5.3).

Comparing the results of the mabs in the ELISA and IFT (Table 5.1 and Table 5.4) it was interesting that the results were only comparable for the two mabs (2E5 1st fusion and 6E8) which gave the highest OD values in the ELISA. This suggested low sensitivity of the ELISA compared to the IFT. Since all mabs that reacted with any virus by ELISA were also positive IFT, the ELISA would appear to be specific but insensitive compared to the IFT.

Analysis of the ELISA and IFT results for each virus showed that there was complete agreement of the two tests only for the orf-11 virus. With the other viruses discrepancy between the tests was due to a reduction in the sensitivity of the ELISA compared to the IFT.

5.2.3 REACTIVITY OF MABS WITH CAPRIPOXVIRUS

All the 27 mabs were sent to the Institute of Animal Health, Pirbright Laboratory, to be screened blind against capripoxvirus. This was necessary because for biosecurity reasons the virus cannot be handled in the UK outside that laboratory.

Results have shown that only one of the twenty-seven mabs (1C7) reacted with capripoxvirus by IFT and also precipitated an approximately 65kDa capripoxvirus protein in RIPA (Dr D Ireland kindly carried out this work). Interestingly 1C7 was the only mab that reacted with all the parapoxviruses studied. Therefore, the 1C7 is recognising a common epitope in the parapox and capripox viruses so far examined.

5.4 DISCUSSION

A notable variation was seen in the cross-reactivity of the mabs against the parapoxviruses by the ELISA and IFT (Table 5.1 and 5.4). The IFT was more sensitive than the ELISA for detecting parapoxvirus, as the number of the mabs which were positive by IFT was greater. This indirectly supports the earlier findings of Koptopoulos *et al.*, (1982) who found ELISA to be less sensitive than an IFT for detecting serum antibody in lambs experimentally infected with orf virus.

Not all the four orf virus strains gave similar binding to the mabs or produced the same OD level in ELISA (Table 5.1), indicating strain variation among the four orf viruses studied. This was in agreement with published reports that demonstrated existence of serological variation between orf strains (Sawhney, 1966; Buddle *et al.*, 1984; Alajeeli *et al.*, 1995) and genetic heterogeneity (Wittek *et al.*, 1980b; Robinson *et al.*, 1982 and 1987; Rafii and Burger, 1985).

Comparison of the mab reactivities with the orf virus strains in the IFT showed the strains orf-11, D1701 and NZ2 reacted with almost all the mabs (Table 5.4). The fact that, fewer mabs reacted with scabby mouth virus suggested that this is an antigenically distinguishable strain. The variable cell culture passage histories of these viruses may have had some influence on their reactivities with the mabs but this would require further study. Wittek *et al.*, (1980b) studied the restriction pattern of two different passages of D1701 (passage 8 and 137). They found limited change between the two isolates, but the general profile of each was very similar.

Indeed reactivity of 23 mabs with Milker's node and 19 mabs with BPSV, was more than the number of the mabs which reacted with scabby mouth virus. Which suggests that parapoxviruses from cattle in Europe are close to the parapoxviruses of sheep in Europe. Further work including larger numbers of BPSV and MNV isolates will be required to see if this panel of mabs can be relied on to distinguish sheep and cattle isolates and aid in the classification of ruminant PPVS. An earlier study by Gassman *et al.*, (1985) based on cross-hybridisation suggested that PPVS from cattle and sheep are clearly distinguishable. Lard *et al.*, (1991) and Czerny *et al.*, (1997) have shown that by IFT and ELISA it is possible to differentiate between individual isolates of orf virus, MNV and BPSV.

From the present study the two mabs 5C3 and 13C10, were found to be specific for orf virus by both IFT and ELISA and it is likely these mabs are recognising a distinctive epitope on the 40kDa protein of orf virus that is not present in the other parapox viruses studied. Other mabs as mentioned in the results are not specific but have overlapping reactivities with parapoxviruses.

Detection of the seal ppv by six mabs (1C7, 7C9, 8D7, 8G5, 10E6, 11B6) and recognition of the squirrel ppv by two mabs (1C7 and 6E11), provides evidence that they are parapoxviruses because until now under the classification of the parapoxviruses by the international committee on the taxonomy of viruses (ICTV) these two viruses were only tentative species of the genus (Esposito *et al.*, 1995). Previously their identification had relied mainly on electron microscopic appearance and IFT with polyclonal antiserum (Wilson *et al.*, 1972; Sands *et al.*, 1984; Osterhaus *et al.*, 1994; Simpson *et al.*, 1994; Nettleton *et al.*, 1995).

Another point to be noticed from the reactivity with seal ppv and squirrel ppv, is that although they are members of the parapoxvirus group, they show considerable divergence from sheep and cattle parapoxviruses.

Swinepox is a member of suipoxviridae with a limited host range (Carn, 1993). Interestingly mabs generated against swinepox detected, by IFT, antigens from four different genera of poxviridae (suipoxvirus, orthopoxviruses, leporipoxvirus and parapoxvirus (Ouchi *et al.*, 1992). Two of their mabs were reacting with swinepox viruses, rabbit fibroma virus and three members of orthopoxvirus (vaccinia, cowpox and ectromelia), while one mab (SP14) was reactive with swinepox viruses, rabbit fibroma virus and orf virus. Another group of the 17 mabs they examined were specific to swinepox viruses and did not react with viruses from any other genera. It would therefore be of interest to test our mabs against other representatives of the poxviridae.

Heterogeneity in the binding ability of the mabs between the PPV is further evidence to support the early suggestion (section 4.3) that these mabs have been produced against different epitopes of the 40 and 65 kDa proteins of orf-11 virus.

It was not surprising to see that the majority of the 40kDa mabs produced the highest OD reading in the ELISA, since, the 40kDa protein is known to be the major envelope protein of orf virus (McKeever *et al.*, 1987; Roberts, 1996) and antibodies against that protein were the first to be detected following orf virus infection. Therefore, the higher OD may have resulted from the presence of a large quantity of this protein in the ELISA antigen or it could be that the 40kDa polypeptide induced antibodies with high affinity.

Almost all the anti-65kDa protein mabs demonstrated inclusion bodies by IFT. Ouchi *et al.*, (1992) found that their mabs reacted with a 65kDa protein and nearly all their other mabs bound to swinepoxvirus reacted with proteins in the pattern of inclusion bodies in the cytoplasm.

All of the previous serological tests which have demonstrated the presence of an antigenic relationship between orf virus and capripoxvirus have been based on cross-reactivity using polyclonal sera (Sharma and Dhanda, 1971; Dubey and Sawhney,

1979; Chand *et al.*, 1994). Cross-reactivity between orf virus and capripoxvirus using a mab has been shown for the first time in this study. The mab 1C7 of the present work which cross-reacted with capripoxvirus, has strengthened the evidence of a serological relationship between the two different genera.

CHAPTER 6
DEVELOPMENT OF MAB BASED VIRUS DETECTION METHODS

6.1 INTRODUCTION

The Enzyme-linked immunosorbent assay (ELISA) is believed to have been developed by the two scientists, Engvall and Van Weemen, around 1970 (Calamel and Lambert, 1988). However, practical applications of the technique were first described by Engvall and Perlmann (1972) and Voller and Bidwell (1975; 1976), since when the test has been widely used.

Most of the ELISA studies done with orf virus were indirect ELISAs for detecting and measuring antibodies in naturally or experimentally infected animals (McKeever *et al.*, 1987; Yirrell *et al.*, 1989; 1994; Housawi *et al.*, 1992).

Several workers have used sandwich or trapping ELISAs for the detection of viral antigen in the diagnosis of animal diseases (Hamblin *et al.*, 1991; Livesay *et al.*, 1993; Libeau *et al.*, 1994; Carn, 1995). Such capture ELISAs have the advantages of, stability of reagents, being easy to operate and being economical (Carn, 1995). Reports of orf being a very acute disease causing very high mortality in lambs and kids (Darbyshire, 1961; Mazur and Machado, 1989) emphasises the need for a diagnostic system that will help in a quick diagnosis. This is especially important in countries where there is a need for a rapid differential diagnosis between orf and the more serious capripoxvirus infections.

The aim of the work described in this chapter was to develop and evaluate ELISAs for the capture of orf antigens present in infected cells grown in the laboratory and in scab material from infected sheep.

6.2 RESULTS

6.2.1 CONJUGATION OF THE MABS

Eight mabs were successfully conjugated with the enzyme horseradish peroxidase (HRP). The mabs were chosen based on two categories, first on the strength of their OD values, and secondly on the protein with which they reacted. Four mabs were against the 40kDa protein (6E8, 9C3, 10E6 and 13C10) and the other four were

against the 65kDa protein (6E2, 7C9, 8B6, 8G5). All the HRP conjugates were reactive when tested against orf-11 ELISA Ag. The end point titre for each of the conjugates was determined (Table 6.1).

Table 6.1: Protein concentration of 8 selected mab ascites and highest dilution of HRP conjugated mab giving an OD \geq 0.2.

Mab ascites	Protein concentration	End dilution of HRP conjugated mabs
6E2	5.760mg/ml	1/24000
6E8	5.680mg/ml	1/768000
7C9	20.017mg/ml	1/3200
8B6	3.900mg/ml	1/6400
8G5	1.654mg/ml	1/12000
9C3	2.147mg/ml	1/12000
10E6	2.740mg/ml	1/1600
13C10	5.610mg.ml	1/6400

6.2.2 COMPETITION ELISA

The competition optical density values were obtained by subtracting the OD value of a mixture of un-conjugated anti-orf virus mab plus HRP - conjugated anti-orf mab from the OD value of an unconjugated anti-BVD virus mab (VPM20) plus HRP conjugated anti-orf virus mab. Optical density values greater than 0.2 at a 1/2000 dilution of HRP conjugated mab provided evidence of competition.

$$\text{Competition ELISA OD} = \left[\begin{array}{c} \text{VPM20} \\ + \\ \text{HRP anti - orf mab} \end{array} \right] - \left[\begin{array}{c} \text{Anti - orf mab} \\ + \\ \text{HRP anti - orf mab} \end{array} \right]$$

e.g. for Table 6.2b:

$$\begin{aligned} \text{Competition ELISA OD} &= \left[\begin{array}{c} \text{VPM20} \\ + \\ \text{HRP 13C10} \end{array} \right] - \left[\begin{array}{c} \text{9C3} \\ + \\ \text{HRP 13C10} \end{array} \right] \\ &= (0.23) - (0.01) \\ &= 0.22 \end{aligned}$$

When the 40kDa mab, 6E8, was used separately with each of the eight HRP - conjugated mabs, it competed only with its homologous HRP - conjugated mab, and not with the other seven mabs (Table 6.2a). Competition of the 9C3 mab with HRP conjugated mabs, revealed that it produced high competition with its homologous HRP- conjugated mab and also competed with mab 13C10 but not with the other mabs (Table 6.2b). Results of the competition of the un-conjugated mab 10E6, showed that it did not compete with the other mabs only with its homologous HRP-conjugated mab (Table 6.2c). The un-conjugated 13C10 mab revealed competition with mab (9C3) (Table 6.2d). The result was almost identical to the reciprocal competition ELISA (Table 6.2b) providing strong evidence that these mabs are competing for the same epitope.

Of the mabs reacting with the 65kDa protein, the mab 6E2 was found to compete with its homologous conjugated mab and with the mab 8G5, but not with any of the other six mabs (Table 6.2e). The 7C9 mab competed only with its homologous conjugated mab (Table 6.2f). Binding of the mab 8B6 showed that it competed with mab 8G5 in addition to the homologous HRP conjugated mab (Table 6.2g). The un-conjugated 8G5 was unusual in that it appeared to compete with two other mabs 6E2 and 8B6 (Table 6.2h).

A summary of the information from the competition studies shows that, of the anti-40kDa protein mabs, the two mabs 9C3 and 13C10 have two way competition with each other (Table 6.3). Of the 65kDa mabs, 8G5 showed evidence of two way competition with both 6E2 and 8B6 but no competition was apparent between 6E2 and 8B6 mabs, suggesting that the 8G5 mab might recognise an epitope overlapped by epitopes seen by 6E2 and 8B6. It would thus appear that the 8 mabs recognise at least 3 epitopes on the 40kDa protein as seen by mabs 6E8, 10E6 and 9C3/13C10; and 3 epitopes on the 65kDa protein as recognised by mabs 7C9, 8G5/8B6 and 6E2/8G5.

Table 6.2a: Competition ELISA of constant non-conjugated Mab 6E8 with eight HRP conjugated mabs. The results are shown as reduction of corrected OD of conjugated Mab and unconjugated Mab from conjugated and negative Mab VPM20.

Conjugated Mab ascites with (HRP)	Dilutions of conjugated mabs			
	200	2000	20,000	200,000
6E2	<0	<0	<0	0
6E8	>2.50	2.26	0.15	0
7C9	0.12	<0	0	0
8B6	0.12	<0	<0	0
8G5	0.22	<0	<0	0.05
9C3	<0	<0	<0	<0
10E6	0.19	0.03	0.01	0
13C10	<0	0.02	0.01	<0

Table 6.2b: Competition ELISA of constant non-conjugated mab 9C3 with eight HRP conjugated mabs. The results are shown as reduction of corrected OD of conjugated Mab and unconjugated Mab from conjugated and negative Mab VPM20.

Conjugated Mab ascites with (HRP)	Dilutions of conjugated mabs			
	200	2000	20,000	200,000
6E2	0.13	<0	<0	<0
6E8	<0	<0	<0	0
7C9	0.13	0	0	0
8B6	0.05	<0	<0	0
8G5	0.13	<0	0.01	<0
9C3	1.19	0.70	0.01	0
10E6	0.03	0	<0	0
13C10	1.92	0.22	0.01	0

Table 6.2c Competition ELISA of constant non-conjugated Mab 10E6 with eight HRP conjugated mabs. The results are shown as reduction of corrected OD of conjugated Mab and unconjugated Mab from conjugated and negative Mab VPM20.

Conjugated Mab ascites with (HRP)	Dilutions of conjugated mabs			
	200	2000	20,000	200,000
6E2	0.16	0.15	0	<0
6E8	<0	<0	0.11	0
7C9	0.26	0.02	0	0
8B6	0.14	0	<0	0
8G5	0.16	0.12	0.01	0.01
9C3	<0	0.13	0	0.01
10E6	1.77	0.52	0	0
13C10	<0	0.03	0.01	0

Table 6.2d Competition ELISA of constant non-conjugated Mab 13C10 with eight HRP conjugated mabs. The results are shown as reduction of corrected OD of conjugated Mab and unconjugated Mab from conjugated and negative Mab VPM20.

Conjugated Mab ascites with (HRP)	Dilutions of conjugated mabs			
	200	2000	20,000	200,000
6E2	0.05	<0	<0	0
6E8	<0	0.02	0.02	<0
7C9	0.08	<0	0	0
8B6	0.23	0	0	0
8G5	0.07	0.03	0	0.01
9C3	1.07	0.67	<0	0.04
10E6	0.09	<0	0.01	<0
13C10	2.0	0.22	0.02	<0

Table 6.2e Competition ELISA of constant non-conjugated Mab 6E2 with eight HRP conjugated mabs. The results are shown as reduction of corrected OD of conjugated Mab and unconjugated Mab from conjugated and negative Mab VPM20.

Conjugated Mab ascites with (HRP)	Dilutions of conjugated mabs			
	200	2000	20,000	200,000
6E2	1.39	0.25	<0	0.01
6E8	<0	<0	<0	<0
7C9	<0	<0	0	<0
8B6	0.02	<0	<0	0
8G5	0.78	0.49	0.03	0
9C3	<0	<0	<0	<0
10E6	<0	0.08	0	<0
13C10	<0	0.15	<0	<0

Table 6.2f Competition ELISA of constant non-conjugated Mab 7C9 with eight HRP conjugated mabs. The results are shown as reduction of corrected OD of conjugated Mab and unconjugated Mab from conjugated and negative Mab VPM20.

Conjugated Mab ascites with (HRP)	Dilutions of conjugated mabs			
	200	2000	20,000	200,000
6E2	0.25	0.03	0	0
6E8	<0	<0	<0	0
7C9	1.33	0.22	<0	0
8B6	0.09	<0	0	<0
8G5	0.28	0.16	0.04	0
9C3	<0	0.15	0.01	0
10E6	<0	0.07	0	0
13C10	<0	0	<0	0.01

Table 6.2g Competition ELISA of constant non-conjugated Mab 8B6 with eight HRP conjugated mabs. The results are shown as reduction of corrected OD of conjugated Mab and unconjugated Mab from conjugated and negative Mab VPM20.

Conjugated Mab ascites with (HRP)	Dilutions of conjugated mabs			
	200	2000	20,000	200,000
6E2	<0	<0	<0	0
6E8	<0	0.03	<0	<0
7C9	<0	0.13	0.01	0
8B6	1.23	0.29	0	0
8G5	0.29	0.37	0.02	0.01
9C3	<0	<0	<0	0
10E6	<0	0	0	0.01
13C10	<0	<0	<0	0.01

Table 6.2h Competition ELISA of constant non-conjugated Mab 8G5 with eight HRP conjugated mabs. The results are shown as reduction of corrected OD of conjugated Mab and unconjugated Mab from conjugated and negative Mab VPM20.

Conjugated Mab ascites with (HRP)	Dilutions of conjugated mabs			
	200	2000	20,000	200,000
6E2	0.40	0.70	0.3	0
6E8	<0	<0	<0	<0
7C9	0.09	0.02	0	0
8B6	0.21	0.28	0	0
8G5	0.51	0.62	0.03	0
9C3	<0	<0	0	0
10E6	<0	0	0.01	0.10
13C10	<0	0.01	<0	0

Table 6.3: Summary of Competition ELISA Results.

Mabs	Competition with homologous mabs	Competition with other mabs
6E2	+	8G5
6E8	+	None
7C9	+	None
8B6	+	8G5
8G5	+	6E2 and 8B6
9C3	+	13C10
10E6	+	None
13C10	+	9C3

6.2.3 DETERMINATION OF BEST COMBINATION OF CAPTURE AND DETECTOR MABS IN AN ORF VIRUS ANTIGEN CAPTURE ELISA

A pilot experiment was set-up to determine the best combination of capture and detector mabs to be used in an ELISA to capture orf virus antigens. Initially, the four anti-40kDa mabs were used individually to capture standard orf-11 ELISA antigen after which any positive binding was detected by HRP conjugated mabs. Only two positive results with OD values ≥ 0.2 were obtained (Table 6.4). 10E6 was the only mab of the anti-40 kDa mabs to have successfully captured orf-11 antigens and then 6E8 - HRP was the only conjugated mab to detect the captured antigen. The other positive result with a low OD was achieved using 6E8 as capture and detector. The 9C3 and 13C10 mabs failed to capture orf antigens as appeared from the low OD they produced.

When the 65kDa non-conjugated mabs were used as capture, they each managed to capture orf-antigens, with 8G5 being the poorest (Table 6.4). The mab 6E2 captured antigens that were detectable by all the HRP mabs against the 65kDa protein and the anti-40kDa mab 6E8 also produced a weak OD. The 7C9 and 8B6 mabs provided a similar sort of capture performance with slight variations in the OD levels recorded by the detector mabs.

Table 6.4 ELISA OD values of pairs of mabs used to capture and detect orf-11 virus antigen. A) Four anti-40kDa mabs used as capture mabs. b) Four anti-65kDa mabs used as capture mabs.

Mab Ascites	Detector mabs conjugated with HRP							
	6E2	6E8	7C9	8B6	8G5	9C3	10E6	13C10
a)								
6E8	0.01	0.33	0.01	0.02	0.00	0.03	0.04	0.05
9C3	0.04	0.03	0.03	0.03	0.04	0.08	0	0.03
10E6	0.03	1.64	0.03	0.04	0.07	0.05	0.0	0.01
13C10	0.08	0.02	0.04	0.03	0.10	0.10	0	0.03
b)								
6E2	1.66	0.34	2.36	2.47	1.41	0.06	0	0.02
7C9	2.26	0.94	0.46	2.24	2.15	0.06	0.02	0.03
8B6	1.90	0.54	0.71	0.25	1.05	0.02	0.01	0.01
8G5	0.34	0.09	0.32	0.20	0.34	0.01	0	0.01

6.2.4 OPTIMISATION OF CAPTURE AND DETECTOR MABS USING ORF-11 ELISA ANTIGEN

Four of the best mab combinations were chosen for further studies. The mab combinations were selected on their OD record and mabs that competed against each other were avoided. Three of the selected combinations were of the anti-65kDa mabs, 6E2, 7C9 and 8B6 as capture with 7C9, 6E2 and 6E2 as detectors. The only anti-40kDa mab system used 10E6 as capture and 6E8 as detector. Optimal OD of each combination was determined by using tenfold dilutions of capture and detector mabs and a 1/800 dilution of orf-11 ELISA antigen (Table 6.5). When the optimal mab dilutions had been determined they were validated using ELISA antigen preparations of 4 different orf viruses.

Table 6.5: The highest ELISA OD value obtained during optimisation of capture and detector mabs against orf-11 ELISA antigen.

Capture mabs	Detector mabs				
	6E2	6E8	7C9	8B6	10E6
6E2	ND	0.36	1.54	1.22	0.01
6E8	0.03	ND	0.03	0.02	0.03
7C9	2.29	0.14	ND	2.00	0.01
8B6	2.19	0.18	0.37	ND	0.02
10E6	0.08	1.56	0.03	0.02	ND

6.2.5 CAPTURE ELISA USING CELL CULTURE GROWN ANTIGENS TREATED WITH NP40

The capture ELISA results of the four orf virus antigens by four different mab combinations showed that 7C9 as capture and 6E2 as detector produced the highest OD values with orf-11, D1701 and scabbymouth antigens, while the other mab combinations were also good. The NZ2 antigen gave the highest OD with 6E2 mab as capture and 7C9 as detector, but again all combinations were good. For all 4 antigens the poorest capture/detector mab combination was with the anti-40kDa 10E6 and 6E8 mabs (Table 6.6).

The data in Table 6.6 was tested by analysis of variance (anova) utilising the Minitab statistics package. A log transformation was used to convert the ELISA reading to normally distributed variables. Anova detected significant differences in response depending on the detector mabs used ($P=0.001$) and on the virus which was used ($P=0.042$).

Table 6.6 Capture ELISA of orf antigens treated with NP40 of orf-11, NZ2, D1701 and Scabbymouth virus preparations

Antigen	Capture non-conjugated mabs			
	6E2	7C9	8B6	10E6
ORF-11	1.49	2.15	1.43	0.67
NZ2	2.29	2.25	2.04	0.83
D1701	2.11	2.34	2.08	1.41
Scabbymouth	1.45	2.09	1.50	0.35
	7C9	6E2	6E2	6E8
Detector HRP-conjugated mabs				

6.2.6 CAPTURE ELISA OF ORF TISSUE CULTURE GROWN VIRUSES BY THE MABS

The capture ELISA mab combinations were next tested on orf virus infected cell culture supernatants without detergent lysis. All the four combinations successfully captured orf-11 virus antigens and produced high OD values when neat cell culture supernatant was tested. The results from all 3 anti-65kDa mab combinations were comparable, with each system readily detecting viral antigen diluted up to 1/100. At the higher dilution only the combination of 7C9 as capture and 6E2 as detector mabs gave an OD >0.2. The reaction with orf-11 virus captured by the anti-40kDa combination of 10E6 and 6E8 dropped quickly upon dilution from neat and although still detectable at 1/10 the result indicated that the quantity of the 40kDa protein in the cell culture suspension was less than that of the 65kDa protein (Table 6.7a).

Capture ELISA of the other orf cell culture grown viruses (D1701, NZ2 and Scabbymouth) produced very similar results to those with orf-11 virus (Tables 6.7b; 6.7c and 6.7d).

When the four tables (6.7a; 6.7b; 6.7c and 6.7d) were simultaneously analysed statistically using analysis of covariance in the Minitab statistics package it was

found that dilution of the antigens had a highly significant effect ($P<0.001$) on the capture ELISA result. A highly significant difference was also noted between the mab combinations ($P<0.001$) but no significant difference was detected between the amount of Ag captured from the different virus preparation ($P=0.808$) indicating that all the cell culture grown viruses had a high amount of orf virus proteins to be captured.

Table 6.7a Capture ELISA of ORF-11 cell culture grown virus

Antigen dilution	Capture non-conjugated mabs			
	6E2	7C9	8B6	10E6
Neat	2.34	2.27	2.38	2.11
1/10 Ag	2.47	2.24	2.28	0.66
1/100 Ag	0.77	2.07	1.10	0.02
1/1000 Ag	0.07	0.28	0.18	0.0
	7C9	6E2	6E2	6E8
Detector mabs (HRP conjugated mabs)				

Table 6.7b Capture ELISA of NZ2 virus cell culture grown virus

	Capture non-conjugated mabs			
	6E2	7C9	8B6	10E6
Neat	2.47	2.28	2.45	2.30
1/10 Ag	2.47	2.29	2.44	1.32
1/100 Ag	0.73	1.63	1.05	0.19
1/1000 Ag	0.08	0.19	0.17	0.19
	7C9	6E2	6E2	6E8
Detector mabs (HRP conjugated mabs)				

Table 6.7c Capture ELISA of D1701 cell culture grown virus

	Capture non-conjugated mabs			
	6E2	7C9	8B6	10E6
Neat	2.47	2.29	2.44	2.20
1/10 Ag	2.46	2.31	2.43	0.57
1/100 Ag	0.54	1.38	0.71	0.05
1/1000 Ag	0.09	0.20	0.15	0.05
	7C9	6E2	6E2	6E8
Detector mabs (HRP conjugated mabs)				

Table 6.7d Capture ELISA of Scabbymouth cell culture grown virus

	Capture non-conjugated mabs			
	6E2	7C9	8B6	10E6
Neat	2.47	2.33	2.45	1.67
1/10 Ag	2.47	2.32	2.44	0.34
1/100 Ag	0.63	1.58	0.85	0.08
1/1000 Ag	0.08	0.21	0.14	0.07
	7C9	6E2	6E2	6E8
	Detector mabs (HRP conjugated mabs)			

6.2.7 EXPERIMENTAL INFECTION OF SPF LAMBS WITH DIFFERENT ORF VIRUS STRAINS

In order to increase the amount of orf virus scabs to be used for capture ELISA four SPF lambs were each scarified on the inner thigh with the cell culture grown viruses. Lamb 29 was infected with orf-11, lamb 37 was infected with D1701, lamb 40 was infected with NZ2 virus and lamb 42 was infected with scabbymouth virus. Blood was collected weekly from day 0 for 4 weeks for serology.

At day 3 post infection three lambs no 29, 37 and 42 all showed hyperaemia at the site of infection, the fourth lamb (40) along with hyperaemia started to develop papules (Figure 6.1). Seven days after infection the lesions of the two lambs 29 and 37 had not progressed further than hyperaemia, but lamb 40 had developed papules and pustules and lamb 42 had produced severe papules and pustules. Four days later, the lesions of the lambs 29 and 37 had almost healed, whereas, some scabs had started to be seen on the lamb infected with NZ2 and more had developed on the lamb infected with scabby mouth virus (Figure 6.2). By day 15, the lesions of lambs infected with orf-11 or D1701 were healed. Small scabs could be collected from the NZ2 lamb and a good quantity was harvested from the scabbymouth infected lamb. Three days later, only small red elevations were seen on lamb 40, while, more scabs could be collected from lamb 42. By day 22 lesions on lamb 40 were barely visible and some areas of lamb 42 had started to heal. Both lambs were completely healed by 28 days after infection.

Figure 6.1a, b, c and d: Colour photographs of skin lesions on 4 SPF lambs each scarified with a different orf virus 3 days previously. Lamb 29 infected with orf-11 showing hyperaemia at site of infection (Fig a); lamb 37 infected with D1701 also showed hyperaemia (b); lamb 40 infected with NZ2 showing hyperaemia as well as papules at the site of infection (c); lamb 42 also showed hyperaemia as a result of scabbymouth infection (d).

A



B



C



D

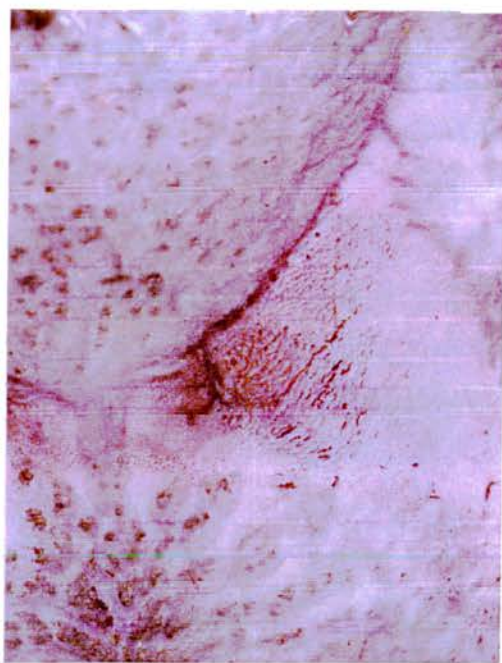


Figure 6.2a, b, c and d: Colour photographs of skin lesions on 4 SPF lambs each scarified with a different orf virus. Results observed on day 11 post infection. The lesion of lamb 29 almost healed (a). Lamb 37 showed a similar result to lamb 29(b). Lamb 40, showed dryness of the papules and pustules and the beginning of scab formation (c). Lamb 42 had scabs over most of the site of infection (d).

A



B



C



D



6.2.8 SEROLOGICAL RESPONSE OF THE SPF LAMBS

Results of testing on sera collected from the SPF lambs for antibody against orf virus by an indirect ELISA demonstrated that all the animals sero-converted (Table 6.8). In general the serological response of the lambs was similar, with antibody detectable in all lambs by day 14 which rose to a maximum recorded OD by day 28. The serological response was related to the clinical lesion: Orf-11 gave the poorest lesion and seroconversion to this virus was the slowest to develop; seroconversion to scabbymouth, however, which had given the most severe clinical lesion was detected first at 7 days post-infection and rose little after 14 days (Fig 6.3).

Table 6.8a Antibody ELISA OD values of 4 SPF lambs infected with a different orf virus

Lamb No.	Infecting virus	Day 0	Day 7	Day 14	Day 21	Day 28
29	Orf-11	0.01	0.01	0.19	1.36	2.01
37	D1701	0.01	0.04	0.68	1.48	1.64
40	NZ2	0	0.04	1.14	1.83	2.37
42	Scabbymouth	0.02	0.19	2.00	2.28	2.33

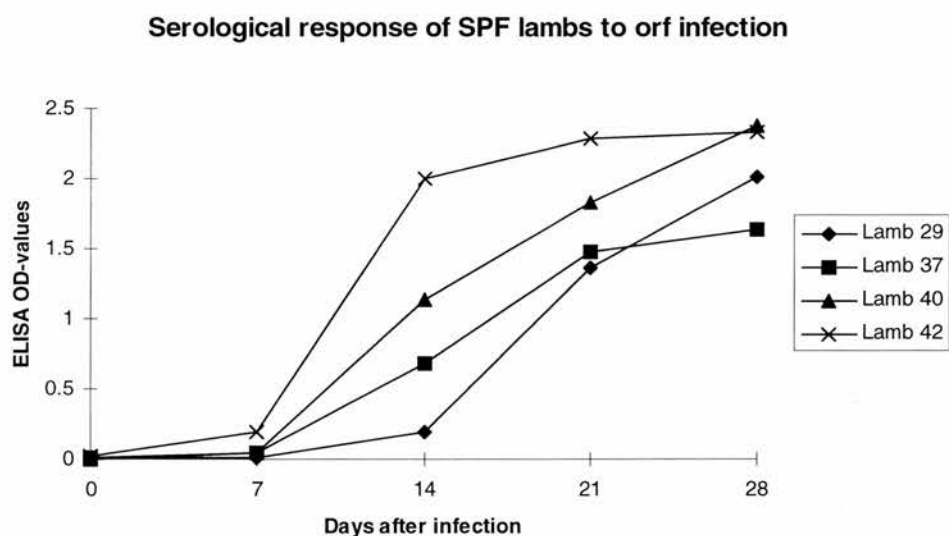


Figure 6.3: Serological response of SPF lambs to experimental orf virus infection (ELISA)

Lamb 29 infected with orf-11 virus, lamb 37 infected with D1701, lamb 40 infected with NZ2 and lamb 42 infected with scabbymouth.

The original OD figures presented in Table 6.8b are unlikely to be normally distributed. To improve the distributional nature of these figures, they were transformed using a function of the form $f(x)=\log(x+1)$, where the logarithmic function is calculated in base 10. The transformed figures were analysed using linear regression in Genstat for Windows, regressing the logged serological response against Day and fitting Virus as a factor in the model. The results are summarised in Table 6.8b.

Table 6.8b Accumulated analysis of variance

Change	d.f.	s.s	m.s.	v.r.	Fpr.
+ Day	1	0.752705	0.752705	90.31	<.001
+ Virus	3	0.052117	0.017372	2.08	0.156
+ Day.Virus	3	0.005177	0.001726	0.21	0.890
Residual	12	0.100017	0.008335		
Total	19	0.910015	0.047896		

This table shows that the basic regression of log-response against Day is highly significant ($P<0.001$). As is clear from Figure 6.3, in all cases the response increases with time. However, the Virus term in the regression (corresponding to any possible differences in intercept between the lines for different viruses) and the Day. Virus term (corresponding to any possible differences in slope) exhibit no evidence to suggest statistical significance. Hence there are no statistically significant differences in response between any of the viruses.

6.2.9A CAPTURE ELISA USING PURIFIED SCAB ANTIGEN

For development of capture ELISA from purified scab antigen, scabs produced by 4 different viruses were studied: MRI reference virus; Scabi-Vax vaccine; Scabbymouth collected from SPF lamb 42, and W2072, a scab from an infected animal in a field outbreak. Virus was purified from each scab and was left untreated or was treated with NP40. Electron microscopy showed that easily detectable amounts of virus particles were harvested from scabs resulting from infection with MRI, Scabi-Vax and Scabbymouth viruses, but only a low number of virus particles were detectable in the W2072 Scab (Table 6.9). Addition of NP40 destroyed the

distinct morphology of the virus and made it difficult to identify the virus (Figure 6.4a and b).

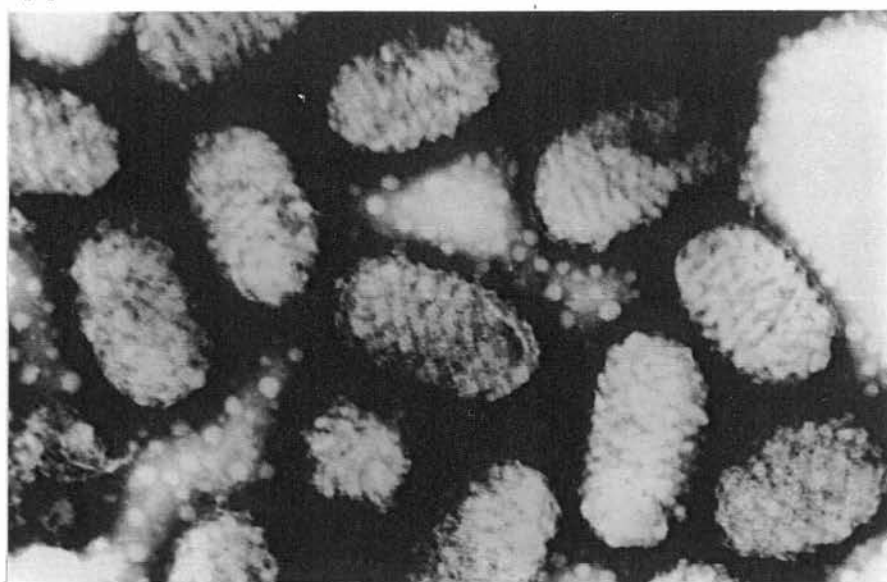
Nonidet NP40 detergent extracts of virus purified from virus scabs were diluted in coating buffer and bound to 96 well plates. In an indirect ELISA the hyperimmune serum (1847) bound to NP40 treated antigens, from all the three purified scabs shown to contain large numbers of viruses, but not the W2072 antigen. A similar result was obtained when the same four antigens were tested in an indirect ELISA with the 6E8 HRP conjugated mab, confirming the presence of detectable orf antigens in the three purified antigens (MRI, Scabi-Vax, and Scabbymouth) (Table 6.9). Surprisingly, none of the 4 anti-65kDa protein HRP conjugated mabs which captured orf antigens from tissue culture grown virus, detected orf antigens from virus purified from scabs. The ELISA OD values were all less than 0.1.

Capture ELISA of orf antigens from virus purified from scabs was successful, using hyperimmune anti-orf serum (1847) as capture, followed by the antigen and then 6E8 HRP conjugated mab as detector. The system managed to capture antigens from all the three different scabs stated. Because no antigen had been detected from scab W2072 in an indirect ELISA with either the hyperimmune serum (1847) or in a direct ELISA with 6E8 HRP- conjugated mab no attempt was made to capture antigens from that purified antigen (Table 6.9).

A similar successful capture ELISA was achieved when 10E6 was used as capture mab and the 6E8 HRP- conjugated mab was used as the detector. Again, purified virus from three of the scabs gave convincing OD's (Table 6.9).

Figure 6.4a and b: Structure of orf virus purified from scab, before treatment with NP40 (a). Structure of the orf virus after addition of NP40 showing loss of outer envelope and loss of morphology (b).

A



B

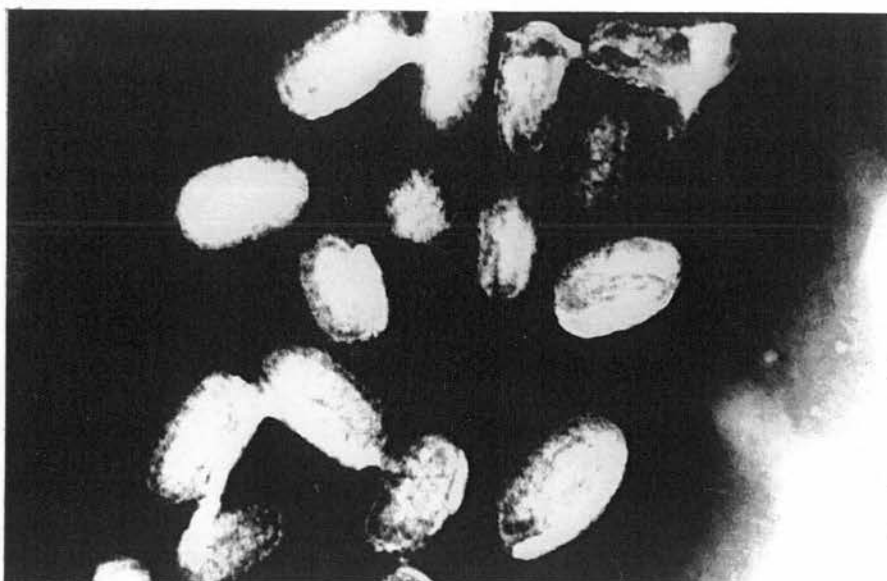


Table 6.9 Indirect ELISA and capture ELISA OD values using NP40 detergent extracts of different orf viruses purified from scabs

Viruses (scab)	EM Result	Reactivity with (1847) hyperimmune	Binding to 6E8 HRP conjugate	Polyclonal anti-orf serum as capture and 6E8 detector	Mab 10E6 as capture and 6E8 detector
MRI	++++v	1.10	0.77	0.90	0.76
Scabi-vax	+++	1.78	1.34	0.92	1.69
Scabbymouth	++++	0.84	0.37	0.93	0.70
W2072	+	0.08	0.06	N/D	N/D

N/D = Not Done

Table 6.10 Capture ELISA OD values of crude 10% suspensions of scabs using polyclonal anti-orf serum as capture and 6E8 as detector mab

Virus scab	Neat	1/50	1/100	1/200
Scabbymouth	0.81	0.50	0.36	0.33
C093 scab	0.05	0.06	0.05	0.05
W1886/2	0.08	0.05	0.08	0.09
C0381	0.40	0.45	0.47	0.40
W1866/1	0.10	0.10	0.11	0.12
W0766	0.24	0.13	0.16	0.18

6.2.9B CAPTURE ELISA OF ORF ANTIGENS FROM 10% SUSPENSIONS OF CRUDE SCAB

Capture of orf antigens from virus purified from scabs demonstrated the potential of capturing orf antigens directly from scabs. Therefore, 10% suspension in PBS plus 1% NP40 of crude scabs were prepared. Antigens from scabbymouth and two other field scabs (C0381 and W0766) were detected with a capture ELISA. No antigens were trapped, however, from any of three other scabs from field cases (C093; W1886/2 and W1866/1) (Table 6.10). The amount of antigens captured was variable as reflected in the OD recorded from one scab to another. The highest result was produced with scabbymouth scab, moderate with C0381 scab and positive but weak with W0766 scab.

The amount of the antigen being captured from scabbymouth suspension appeared to fall gradually coinciding with dilution. However, antigens trapped from C0381 scab remained about the same in the four dilutions (Table 6.10), suggesting a high level of antigen in this scab suspension.

Although, using the two mabs 10E6 as capture and 6E8 as detector was able to capture orf Ag from purified scabs (Table 6.9) they did not capture orf from crude 10% suspension. The amount of scab protein bound and detected with negative mab VPM20 gave nearly similar OD to that when 10E6 was used as capture, again suggesting non specific interference in the scab suspension bound to the capture mabs. No success was obtained when other mabs were used to study capturing of orf antigens from scabs.

6.3 DISCUSSION

As expected, in the competition ELISA of the mabs studied, each of the conjugated mabs was found to compete with its homologous mab, leading to reduction in OD level which can be attributed to many antigen binding sites being occupied with non-conjugated mab leaving few sites for the conjugated mab to bind to.

Competition with heterologous mabs was seen between the two mabs 9C3 and 13C10 showing that these two mabs were reacting with the same immune determinants. The pair wise competition also seen between 6E2 and 8G5 and 8B6 and 8G5 have evidently shown that the epitope recognised by the mab 8G5 has shared antigenic determinants with the two epitopes recognised respectively by 6E2 and 8B6. Strikingly, the determinant seen by 6E2 and 8B6 was not identical, a situation which has also been described in mabs competing for Bovine Herpes Virus 1 (BHV-1) epitopes (Collins et al 1984).

Clearly, the competition studies, like the results of Chapter 4, demonstrated discrimination between the antigens of the 40 and 65kDa proteins, apart from the weak competition produced by 6E8 with some of the 65kDa mabs. Very recently, Czerny *et al.*, (1997) described three distinct antigenic sites in orf virus by competition ELISA, but none the less the three sites were found to be closely related according to some extent of inhibition between the mabs.

For some unknown reasons, three of the 40kDa mabs (6E8, 9C3 & 13C10) lacked capturing ability, however, the mab 10E6 and all the 65kDa mabs (6E2, 7C9, 8B6 and 8G5) were able to capture orf virus antigens. It is unlikely that the 9.6 pH carbonate bicarbonate buffer had effect on the mabs since other mabs had captured antigens. Because the mabs which managed to capture orf antigens were of similar isotype to those that did not, the isotype can be excluded as another cause. My explanation therefore, is that such behaviour is due to the structure of those mabs; especially in the sequence of their FC regions which prevent them from binding.

It was evident from the ELISA OD levels scored that there was significant variation between the mab combinations used to capture antigen treated with NP40, which reflected differences in the amount of the Ag being captured from different virus preparations. This has been supported by the statistical results using analysis of variance which revealed significant differences between the mabs as well as significant difference between the four viruses studied.

Absence of significant difference between tissue culture propagated viruses in the capture ELISA ($P=0.808$) with the analysis of the antigen of the same viruses treated with NP40 indicated all orf viruses released high quantities of the 65 and 40kDa during *in vitro* replication.

Of the two proteins captured by the mabs, in both NP40 treated antigens or tissue culture grown virus, the quantity of the 40kDa protein was apparently less than that of 65kDa protein, which would explain why the majority of the mabs produced were against the 65kDa, in accordance with Schindler (1995).

Experimental orf virus infection with 4 cell-culture grown viruses produced cutaneous orf lesions similar to those previously described (McKeever *et al.*, 1988; Jenkinson *et al.*, 1992; Zamri-Saad *et al.*, 1992; Housawi *et al.*, 1993). Two of the orf virus strains Orf-11 and D1701 when applied to scarified skin of SPF lambs produced no more than hyperaemia in the site of infection demonstrating that the two viruses have become very attenuated following *in vitro* adaptation. In field trials Zach, (1979) and Mayr *et al.*, (1981) have used D1701 to vaccinate sheep. Because the sheep were infected subcutaneously they did not report occurrence of any skin lesions on the animals. Contrary to the Orf-11 and D1701 strains, scabbymouth, produced typical aggressive orf picture, as reported by Pye (1990) and Nettleton *et al.*, (1996a) indicating that, although, scabby mouth has been passaged in tissue culture it has retained its virulence for sheep and produces vigorous lesions.

As reported elsewhere, all the sheep seroconverted after experimental infection, including those which did not show any progress in their skin lesions (McKeever *et al.*, 1987; Yirrell *et al.*, 1989; Nettleton *et al.*, 1996b). However, detection of high antibodies in the sheep does not mean they are protected from subsequent infection, since immunity against orf is described to be cellular (Robinson and Balassu, 1981; McKeever *et al.* 1987). An observation which has been supported by the findings of Reid and other (unpublished data) that sheep infected with Orf-11 virus are

susceptible to challenge infection with virulent virus despite the presence of humoral antibodies.

Despite the remarkable success in capturing orf antigens with the combinations of the 65kDa mabs from both orf virus ELISA Ag treated with NP40 and from tissue culture grown virus, none of these combinations resulted in evidence of captured orf antigens from purified scab or crude scab suspensions. This may be related to several factors: cell cultures offer the optimum condition for the 65kDa protein to be released during the viral replication; the 65kDa protein is less stable in scab or it occurs in very small amounts in the scab specimens.

Treatment of purified Scab Ag with NP40 was useful during Ag preparation (Figure 6.4), resulting in disruption of the virus particles. Madboly et al (1987), have described better rinderpest antigen extraction when the infected cells are treated with NP40. Viral antigen from three of the four purified scabs examined (Table 6.8) were successfully captured, absence of previous published data on the use of capture ELISA for orf virus or any parapoxvirus make it difficult to compare with other studies. Czerny *et al.*, (1997) stated capturing of orf virus Ag, but provided no other detailed information of the method used or the results.

Reports are controversial over the presence of orf virus in scabs. Many authors described that orf virus in scabs is stable for months to years (Livingston and Hardy; 1960; Robinson and Balassu, 1981; McKeever, 1984). On the other hand Romero-Mercado *et al.*, (1973b), failed to detect orf virus antigen in naturally shed scabs. Considering the results of the crude scab capture ELISA, the three samples (Table 6.10) which failed to be captured may have had very low virus titre. One must not forget also that the source of the scab could have come from mild or severe outbreak, recent or old as important factors, with the best amount of the antigen trapped from Scabbymouth Scab. A relevant piece of work was published by Carn (1995) who captured capripox antigen from tissue culture virus. When she tested clinical material

from infected sheep/goats, however, antigen was trapped from only four of the twelve samples examined, supporting the work described in this chapter.

It is good to see the system developed working with Scabbymouth Scab, C0381 and W0766 virus antigen, but further work is required to study scabs of recent and old origin, severe and mild outbreak history to substantiate these preliminary findings. In addition examination of the trapping system developed is needed to determine specificity against some of the diseases which demand differentiation from orf such as sheeppox, goatpox, and blue tongue.

CHAPTER 7
GENERAL DISCUSSION

7.1 HIGHLIGHT ON MONOCLONAL ANTIBODIES AGAINST ORF VIRUS AND THE PROTEINS THEY IDENTIFIED

The studies detailed in chapter 3 reported the production of mabs against orf virus. Mabs against orf virus have also been obtained by other groups in USA (Lard *et al.*, 1991), France (Schindler, 1995) and Germany (Czerny *et al.*, 1997). Two of these groups reported their findings after this project had been initiated and provide a point of comparison for the work.

Neutralisation is a major factor in the characterisation of the specificity of antibodies and in understanding the nature of immune reactions. All the 27 mabs described had no neutralising activity against orf virus, similar to the mabs produced by Lard *et al.*, (1991) and Schindler (1995). In fact only one mab (4D9) has been described to have neutralised orf virus (Czerny *et al.*, 1997). In orthopoxviruses several authors have demonstrated neutralisation by mabs, for instance, in vaccinia (Rodriguez *et al.*, 1985; Ichihashi and Oie, 1988; Czerny and Mahnel, 1990; Gordon *et al.*, 1991, Chang *et al.*, 1995), cowpoxvirus (Kitamoto *et al.*, 1987; Czerny *et al.*, 1994a). However, no neutralisation was obtained from mabs raised against monkeypoxvirus or ectromelia virus (Czerny 1994a).

Interestingly, Rodriguez *et al.*, (1985) produced three neutralising mabs against VV (C3, B11 and mab F11) and each of these reacted with a 14kDa protein. The mab C3 was found to prevent VV uncoating and the other two mabs acted similarly. Moreover, these three mabs were also capable of neutralising cowpox virus and rabbitpox virus, indicating conservation of the 14kDa amongst orthopoxviruses and of the practical importance of this protein. The gene encoding the 10kDa protein in the orf virus NZ2 that is homologous to the 14kDa protein of VV has been identified (Naase *et al.*, 1991). No mabs against this orf virus protein have so far been produced. Whether mabs or polyclonal sera against the 10kDa orf virus protein would provide similar neutralisation as seen with VV by the mab C3 should be explored.

Neutralising antibodies from animals which had natural or experimental orf virus infections have been demonstrated (Poulain *et al.*, 1972; Jan *et al.*, 1978; Czerny *et al.*, 1997).

Unlike orf virus, VV has been completely sequenced and the genes encoding many of its proteins identified (Groebel *et al.*, 1990). A number of mabs have been raised against different orf virus proteins with 14kDa, 21, 22, 39, 40, 41, 66kDa (Lard *et al.*, 1991; Schindler, 1995; Czerny *et al.*, 1997), but the genes encoding these proteins have yet to be conclusively identified and the roles of the proteins remain obscure.

Despite the fact that up to 35 proteins were resolved from orf virus, only a limited number were found to be immunogenic (Balassu and Robinson, 1987; McKeever *et al.*, 1987; Chand *et al.*, 1994). Of the two immunodominant proteins mentioned in chapter 4, the 40kDa has been cloned and sequenced (Roberts, 1996). The sequence of the gene has been designated as F1L which showed homology to the H3L gene of VV encoding a 35kDa protein. More information about F1L gene was described in Chapter 4.

The vaccinia H3L gene from the Livp strain was found to code for a P35 protein which is considered to be a major envelope protein (Zinoviev *et al.*, 1994). They were able to extract the 35kDa protein using non-ionic detergent NP40 and cyanogen bromide. Up to four homogeneous peptides were recognised from the protein and their N terminal amino-acid (aa) sequences were determined. They were found on the aa sequence at residues 44-63, 144-149, 154-165 and 224-238 of the H3 open reading frame of the HindIII H fragment of the VV genome (Zinoviev *et al.*, 1994).

There was some earlier controversy over the identity of the gene expressing the immunodominant 35kDa VV major envelope protein. Gordon *et al.*, (1988) and Muravlev *et al.*, (1990), were of the opinion that the H6 gene and the A2 gene encoded the P35, and it was not until, Zinoviev *et al.*, (1994) cloned and studied the products of the three genes H3, H6 and A2 that their roles were confirmed. Based

on, immunochemical analysis, the H3 gene was found to be the major envelope product of VV (Livp strain). The H3L gene was noted to be highly conserved and structurally similar between VV and other orthopoxviruses (Rosel *et al.*, 1986; Groebel *et al.*, 1990; Zinoviev, *et al.*, 1994).

The exact identity of the 65kDa is still unknown, due to absence of any previous information about that protein. Nevertheless, the gene has been localised to a 14kb Kpn1A and Sau3 fragment with co-ordinates 75.4-89.4Kb on the OV restriction map (Mercer *et al.*, 1997). Primary sequencing analysis (T Fitzgerald, unpublished data) and alignment with the VV sequence has shown evidence of homology with the VV gene known as A3L gene and also known as 4b encoding as core polypeptide (Johnson *et al.*, 1993). The gene 4b was found to express the major core 62kDa polypeptide and its precursor known as P 4b to express a 74kDa product (Rosel and Moss, 1985). The P 4b is located in the left end of HindIII A fragment and its sequence encodes for a protein of 644 amino-acids.

Previous studies on parapoxviruses by Thomas *et al.*, (1980); McKeever *et al.*, (1987) and recently by Czerny *et al.*, (1997) have all reported a core protein which pellets on removal of the envelope proteins. Czerny and his co-workers reported detection of two bands with molecular weights of 66 and 22kDa from the pelleted core material, but otherwise none of the three studies has provided any detailed data to support their findings. The results presented in chapter 4 have indicated the importance of the 65kDa as well as the 40kDa antigens of OV. If subsequent work confirmed that the 65kDa is a core protein, it will be the first core polypeptide to be identified from orf virus and parapoxviruses in general.

Recently Senkevich *et al.*, (1997) have described sequencing the molluscum contagiosum virus and identification of 182 encoded proteins. Strikingly 105 of the 182 were found to have counterparts in Orthopoxvirus. A homologue to the gene expressing P37 of VV was among the identified proteins, and thus the P37 is found

in four poxviruses namely VV, fowl pox, orf and molluscum contagiosum (Calvert *et al.*, 1992; Sullivan *et al.*, 1994; Senkevich *et al.*, 1997).

It has been described, using transcriptional and translational mapping of VV that early and late genes are distributed along the length of the DNA molecule (Wittek *et al.*, 1980a) except at both ends of the genome which are occupied by tandem repeats. Since orf virus was found to have tandem repeats (Fraser *et al.*, 1990), the transcriptional and translational orf genes may also be presumed not to occur at the tandem repeat sites (Fleming, personal communication).

A surprising phenomenon was noticed with the reactivity of the 6E11 mab on western blotting and radioimmunoprecipitation. In western blots the 6E11 clearly detected a 40kDa protein of orf virus and in RIP it bound to a 50kDa protein. None of the other 26 mabs reacted in the same way. The actual reason for that requires further study. However, mabs reacting with one protein of non-cpe a noncytopathic border disease virus, (BDV) was found to react with two proteins of a cytopathic biotype of BDV (Dutia *et al.*, 1990). With cowpox virus a mab was produced that detected a 160kDa protein of A-type inclusion bodies (ATIB) of LB red or LB white strains and recognised an 80kDa protein of Amsterdam 53, 58 and 60 strains (Kitamoto *et al.*, 1986a). In this case the 160kDa protein is thought to be a dimer of the 80kDa protein.

In the reactivity of the anti-40kDa protein mabs against the new recombinants 380 and 436 expressing the 40kDa product, only three mabs 2E5 (1st fusion), 8D7, 10E6 were reactive with the two recombinants, the other five showed no reactivity as described in Section 4.3. Since four of the mabs had reacted with the original recombinants 245/247 failure of mab 6E8 to react with the 380 and 436 recombinants was the most surprising. Failure of this mab, and the other 40kDa mabs to react, could be due to their recognising epitopes of the 40kDa protein expressed *in vivo* but not those expressed by the 380 and 436 VVOV recombinant machinery.

7.2 CROSS - REACTIVITY OF MABS BETWEEN ISOLATES OF PARAPOXVIRUSES AND WITH CAPRIOPOXVIRUS

Because of their absolute specificity for individual epitopes, mabs are considered as the best kind of antibody for antigenic analysis (Tizard, 1987). That has clearly been demonstrated in Chapter 5. In the cross-reactivity study some mabs (5C3 and 13C10) were specific for orf virus, 1C7 detected all parapoxviruses examined and other mabs showed variability in their reactivity to different parapox viruses (Table 5.2). In a similar approach, Lard *et al.*, (1991) were able to differentiate three parapoxviruses. Their mab 1A1 reacted strongly with orf virus and weakly with MN virus, but not with BPS virus. Surprisingly, none of their mabs reacted only with orf virus. Despite the fact their mabs were raised against orf virus all reacted with MN virus, supporting the view mentioned earlier that parapoxviruses of bovine origin are close to those of ovine origin. However at the molecular level, little resemblance was found in the restriction patterns of parapoxviruses from sheep and cattle (Wittek *et al.*, 1980b; Gassmann, *et al.*, 1985). Even the two cattle viruses MNV and BPSV were clearly distinguishable from each other (Gassmann *et al.*, 1985).

Poxviridae consist of eight genera, which are: orthopoxvirus, parapoxvirus, avipoxvirus, capripoxvirus, leporipoxvirus, suipoxvirus, molluscipoxvirus and yatapoxvirus (Binns and Smith, 1992; Franki *et al.*, 1992). The parapoxvirus genus has four definite members - orf virus (OV), Milker's node virus (MNV), Bovine papular stomatitis virus (BPSV) and Red Deer parapoxvirus (Esposito *et al.*, 1995; Robinson and Mercer, 1995). Tentative species include: camel parapoxvirus (Auzdyk disease), chamois contagious ecthyma virus, seal parapoxvirus and squirrel parapoxvirus (Fenner *et al.*, 1987; Binns and Smith, 1992).

The positive reactivity of the mabs 1C7, 7C9, 8D7, 8G5, 10E6 and 11B6 with seal ppv and mabs 1C7 and 6E11 with the squirrel ppv provides evidence to support the claim that these two viruses are parapoxviruses (Binns and Smith, 1992). Previously only morphological evidence was available with both viruses having the unique

parapoxvirus pattern on electron microscopic examination (Sands *et al.*, 1984; Nettleton, *et al.*, 1995).

Ouchi and his co-workers (1992) generated mabs against swinepox virus. Their mabs had interesting cross-reactivity among poxviruses. One mab detected orf, rabbit fibroma and swinepox viruses; two other mabs reacted with VV, cowpox, ectromelia, rabbit fibroma as well as swinepox viruses, showing existence of epitope conservation among the poxvirus family.

Inclusion bodies are often seen in immunofluorescent antibody tests on poxvirus infected cells (Kitamoto *et al.*, 1986b; Ouchi *et al.*, 1992). These inclusion bodies were reported to have a limited role in the virus multiplication, and represent aggregates of viral multiplication (Kato *et al.*, 1959a+b). Thus the inclusions bodies described in the present study have an unknown role apart from showing the site of virus infection.

All orthopoxviruses are reported to produce inclusion bodies (Fenner, 1990). Using mabs reacting with A-Type (ATIB) inclusion bodies of cowpoxvirus Kitamoto *et al.*, (1986a) revealed the presence of some antigenic variation in ATIB in different orthopox viruses. Some mabs reacted with cowpoxvirus (CPV) and ectromelia virus (EV) ATIB and others were detecting ATIB of CPV, EV and VV. Therefore, these mabs could be used for the differentiation and diagnosis of orthopoxviruses.

In molluscum contagiosum virus three genes were found to encode for ATIB, designated as MC 130L, MC 131L and MC 133L (Senkevich *et al.*, 1997). Surprisingly, two of these genes, MC 131L and MC 133L, had homology with C-terminal domains of the 14kDa protein of vaccinia virus (WR strain) (Senkevich *et al.*, 1997).

Evidence for the existence of cross-reactivity between capripoxvirus and parapoxvirus has been reported (Bennett *et al.*, 1944; Sharma and Dhanda, 1971;

Dubey and Sawhney, 1979; Subba Rao and Malik, 1979; Chand *et al.*, 1994). However, Chand *et al.*, (1994) found that antibodies against a 32kDa protein of capripoxvirus did not react with any orf virus protein. The gene encoding for the Kenya sheeppox-1 (KS-1P32) was cloned and expressed in E coli as a fusion protein with glutathione - S - Transferase (GST) (Carn *et al.*, 1994). Interestingly, the GST-P32 fusion protein could be used to protect against virulent cpv challenge (Carn *et al.*, 1994). It would be interesting to know whether a similar approach with orf virus would provide good protection.

In the screening of the orf virus mabs against cpv (chapter 5) 1C7 was the only mab which reacted with a 65kDa protein of capripoxvirus, indicating similarity in the determinants and the molecular weight of the two proteins. It is not known if these two proteins have the same role in capripoxvirus and parapoxvirus. A cpv protein of very similar molecular weight (64kDa) has been described (Kitching *et al.*, 1986a). Unlike the orf 65kDa protein which is thought to be a major core protein (Fitzgerald, data unpublished), the 64kDa protein of capripoxvirus is described as being a major envelope protein (Kitching *et al.*, 1986a).

7.3 EXPERIMENTAL INFECTION OF SHEEP WITH TISSUE CULTURE PROPAGATED ORF VIRUS

As discussed in chapter six, typical orf lesions were observed in lambs infected with scabbymouth virus. Papule, pustule and scabs were each noted as previously described (McKeever *et al.*, 1988; Jenkinson *et al.*, 1990b). Although, there was interference by collecting scab material during the infection, the complete healing of the lamb infected with scabby mouth virus was around day 28 post-infection. In general the course of orf disease is described as being between 4 and 5 weeks (Robinson and Balassu, 1981; McKeever *et al.*, 1988; Yirrell *et al.*, 1989).

Studies by many investigator have shown orf virus has the capability to reinfect and replicate in the skin of previously infected animals (McKeever *et al.*, 1988; Jenkinson *et al.*, 1990b; Haig *et al.*, 1996c), despite the presence of cellular immunity

and humoral antibodies. The exact reason for that is not fully understood. Nevertheless, the virus has been found to possess a number of genes namely an anti-GM-CSF inhibitory gene as well as VEGF, E3L and 1L10 genes (McInnes and Haig, 1991, Lyttle *et al.*, 1994; Haig *et al.*, 1996c; Fleming *et al.*, 1997). These genes are thought to be responsible for orf virulence and the ability of the virus to temporarily avoid host immunity (Haig *et al.*, 1996 b).

The importance of the experimental infections studied here, is that, the scabby mouth virus, still has the ability to produce very aggressive skin lesion, despite being grown in tissue culture. In contrast the other 3 cell culture adapted viruses produced few clinical signs although they did appear to replicate as judged by seroconversion of the infected sheep.

7.4 DIAGNOSIS OF ORF VIRUS

The diagnosis of orf has been demonstrated by several methods:- by reproduction of the disease in susceptible animals (Howarth, 1929; Samuel *et al.*, 1975; Watt, 1982), detection of the virus by EM (Nagington and Whittle, 1961; Robinson and Petersen, 1983; Buttner *et al.*, 1990) or by serological identification (Sharma and Bhatia, 1959; Romero-Mercado *et al.*, 1973a; Erickson *et al.*, 1975; Azwai *et al.*, 1995; Chin and Petersen, 1995; Nettleton *et al.*, 1996a).

Variability in the accuracy and sensitivity of some of the methods has been described (Romero-Mercado *et al.*, 1973a,b). Other techniques like SNT are dependent on tissue culture adapted virus, which can be difficult as viruses do not readily adapt to *in vitro* growth systems. Difficulties with each of these methods have lead researchers to look for new diagnostic approaches. Lately Buttner *et al.*, (1996) reported preliminary diagnosis of orf using polymerase chain reaction (PCR) with primers for the gene encoding the 10 kDa fusion protein. In the present work presented in chapter six, the capture system developed was successfully able to detect orf virus antigens in tissue culture and to a lesser extent in scabs. These results were

similar to the polyclonal antibody capture systems developed for capripox diagnosis (Carn, 1995).

Although, the contribution of the work presented in thesis may not have been planned directly for eradication of orf disease, more further work will be helpful by providing quick and accurate diagnosis, thus leading to an improvement in the management and control of the disease. Moreover, the capture technique developed is easy to use, affordable for developing countries or anywhere where the disease has been reported.

7.5 CONCLUSION

- Monoclonal antibodies of variable affinity and isotype have been produced
- The Monoclonal antibodies recognise a minimum of two orf virus proteins, one of which is the 40kDa major envelope protein.
- Differentiation between parapox viruses by the mabs is feasible.
- Capture of orf virus antigens from tissue culture grown virus and scab has been demonstrated.

7.6 FUTURE WORK

- Use the mabs to investigate the structure and function of the 40kDa major envelope protein and the 64kDa putative major core protein and to determine the relationship between the 40kDa and 50kDa proteins identified by mab 6E11.
- Use cross-reactivity of the mabs to investigate a wider number of BPS and Milker's node viruses.
- Study the specificity of the capture system developed against some of the sheep disease, that could be considered important for differential diagnosis from orf such as sheep and goat pox; bluetongue and FMD.
- Study scab samples from a wide range of orf outbreaks including recent and old, mild and severe.

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APPENDIX
REAGENTS AND SOLUTIONS

REAGENTS

1. **1 X RPMI medium without L-Arginine,L-Cystine,L-Glutamine,I-Inositol, L-Leucine,L-Methionine, Glucose**
Gibco
Cat No: 51871-010
2. **100 X concentrated arginine**
Gibco
Cat No: 31008-014
3. **100 X concentrated cystine**
Gibco
Cat No: 21033-014
4. **100 X concentrated glucose**
Gibco
Cat No: 39002-019
5. **100 X concentrated I- inositol**
Gibco
Cat No: 23390-016
6. **100 X concentrated leucine**
Gibco
Cat No: 21077-011
7. **10M hydrochloric acid HCl mwt 36.46**
Fisons
Cat No: H/1200/PB17
8. **10x 199 MEDIUM (Earle's modified salts, without NaHCO₃ with glutamine)**
Gibco
Cat No: 21183-033
9. **2-Mercaptoethanol C₂H₆OS mwt 78.13**
Sigma
Cat No: M 7154
10. **30% W/V acrylamide/0.8% W/V BIS acrylamide 37.5:1 (Easigel)**
Scotlab
Cat No: SL-9207
11. **50ml centrifuge tubes**
Gibco
Cat No: 373660

12. **Ammonium persulphate** $(\text{NH}_4)_2\text{S}_2\text{O}_8$ mwt 228.2
Sigma
Cat No: A9164

13. **Aprotinin (from bovine lung)** mwt 6,500
Sigma
Cat No: A4529

14. **Bromophenol blue (sodium salt)** $\text{C}_{19}\text{H}_9\text{Br}_4\text{O}_5\text{Na}$ mwt 691.9
Sigma
Cat No: B 7021

15. **Cassette GRI Side Action Lever Lock 18 x 24 cm**
GRI
Cat No: 400/S4

16. **Citric acid** mwt 210.14 $\text{C}(\text{OH})(\text{COOH})(\text{CH}_2.\text{COOH})_2.\text{H}_2\text{O}$
BDM
Cat No. 100813M

17. **DAB - 3,3' Diaminobenzidine (tetrahydrochloride)** mwt 360.1 $\text{C}_{12}\text{H}_{14}\text{N}_4.4\text{HCl}$
Sigma
Cat No: D5637

18. **DePeX mounting medium 'Gurr'**
BDH
Cat No. 36125 2B

19. **Deoxycholic acid Sodium salt** $\text{C}_{24}\text{H}_{39}\text{O}_4\text{Na}$ mwt 414.6
Sigma
Cat No: D6750

20. **Developer**
HA WEST
Cat No: D-19

21. **Di-sodium hydrogen orthophosphate anhydrous** mwt 141.96 Na_2HPO_4
Fisons
Cat No. S/4520/53

22. **DMSO (Dimethyl sulfoxide)** mwt 78.13 $\text{C}_2\text{H}_6\text{SO}$
Sigma
Cat No. D-5879

23. **Donkey anti sheep/goat IgG horseradish peroxidase**
SAPU
Cat No. S084-201

24. **ELISA plates M129A**
 Dynatech
 Cat No. 655070

25. **Foetal bovine serum**
 Bioclear
 Cat No: 37187

26. **FUJI RX film 18 x 24cm** Box 100 non interleaved sheets
 GRI
 Cat No: JTS010

27. **Glycerol** $\text{CH}_2\text{OH}.\text{CHOH}.\text{CH}_2\text{OH}$ mwt 92.1
 BDH
 Cat No: 10118

28. **Glycine** mwt 75.07 $\text{C}_2\text{H}_5\text{NO}_2$
 Fisons
 Cat No:G/0800/48

29. **Hanks balanced salt solution without sodium bicarbonate**
 Gibco
 Cat No: 61200-093

30. **HAT 50X concentrate**
 Flow Laboratories
 Cat No. 16-808-49

31. **HT 50X concentrate**
 Flow Laboratories
 Cat No. 16-809-49

32. **Hepes (N-(2-Hydroxyethyl)piperazine, N'-(2-ethanesulphonic acid)** $\text{C}_8\text{H}_{18}\text{N}_2\text{O}_4\text{S}$
 mwt 283.3
 Sigma
 Cat No: H3375

33. **Isopropanol** (2-Propanol) $\text{C}_3\text{H}_8\text{O}$ mwt 60.10
 Sigma
 Cat No: I- 9516

34. **Kodak unifix**
 HA West
 Cat No: 501 1036

35. **L-glutamine** mwt 146.1 $C_5H_{10}N_2O_3$
Sigma
Cat No: G3126

36. **L-[^{35}S] methionine** *in vivo* cell labelling grade 370MBq/ml
Amersham
Cat No: SJ1015

37. **Methanol** $CH_3.OH$ mwt 32.04
Fisons
Cat No: M4000/17

38. **Mini protean II dual slab cell**
Biorad
Cat No: 165-2940

39. **Nonidet P40**
Sigma
Cat No: N-6507

40. **OPD - O-phenylenediamine (1,2 - Benzenediamine) dihydrochloride** mwt 181.1
 $C_6H_8N_2.2HCl$
Sigma
Cat No. P-1526

41. **Phenol red** mwt 354.4 $C_{19}H_{14}O_5S$
Fisons
Cat No: P/2420/46

42. **Phenylmethyl sulfonyl fluoride (PMSF)** $C_7H_7FO_2S$ mwt 174.2
Sigma
Cat No: P7626

43. **Polyethylene glycol 1500 (PEG 1500)**
Boehringer Mannheim, Biochemica
Cat No. 783 641

44. **Ponceau red**
Sigma
Cat No: P7767

45. **Potassium dihydrogen orthophosphate** mwt 136.09 KH_2PO_4
Fisons
Cat No. P/4800/53

46. **Potassium chloride** mwt 74.56 KCl
Fisons
Cat No. P/4280/53

47. **Protein G-agarose from Group C Streptococcus sp.**
Sigma
Cat No: P2294

48. **Rainbow ^{14}C molecular weight markers (mwt 14,300-200,000 Da)**
Amersham (1.85kBq/ml)
Cat No: CFA 626

49. **RPMI - 1640 - powdered medium**
Hyclone
Cat No. B-0304-BB

50. **SDS-page prestained standards Broad range(7,300-204,000 kd)**
Biorad
Cat No: 161-0318

51. **Sheep anti mouse IgG horseradish peroxidase**
SAPU
Cat No. S081-201

52. **Sodium bicarbonate (Sodium Hydrogen Carbonate)** mwt 84.01 NaHCO_3
Fisons
Cat No: S/4240/53

53. **Sodium carbonate anhydrous** mwt 105.99 Na_2CO_3
Fisons
Cat No. S/2920/53

54. **Sodium chloride** mwt 58.44 NaCl
Fisons
Cat No. S/3160/60

55. **Sodium dodecyl sulphate** $\text{C}_{12}\text{H}_{25}\text{OS.Na}$ mwt 288.38
Sigma
Cat No: L4509

56. **Sodium hydroxide** mwt 40 NaOH
Fisons
Cat No: S/4920/53

57. **Sodium hydrogen carbonate** mwt 84.01 NaHCO_3
Fisons
Cat No. S/4240/53

58. **Sucrose** $C_{12}H_{22}O_{11}$ mwt 342.30
Fisons
Cat No: S/8600/60
59. **Sulphuric acid conc.** H_2SO_4 mwt 98.07
May & Baker
Cat No. L741
60. **Temed (N,N,N,'N' - Tetramethylethylenediamine)** $C_6H_{16}N_2$ mwt 116.2
Sigma
Cat No: T-7024
61. **Tris (hydroxymethyl) aminomethane** $C_4H_{11}NO_3$ mwt 121.1
Boehringer Mannheim
Cat No: 708976
62. **Tryptose phosphate broth mwt**
Gibco
Cat No: 15125-024
63. **Tween 80 polyethylene sorbitan monolaurate**
Sigma
Cat No. P-1754

SOLUTIONS

Cell culture/ELISA

RPMI - 1640

1X RPMI 1640	500mls
0.1M Glutamine	5mls
80g/l Sodium Bicarbonate	13.75mls

Laying Away Media (Liquid nitrogen)

RPMI 1640 + 10% Foetal Bovine Serum (FBS)	4mls
Foetal Bovine Serum	5mls
Dimethyl sulfoxide	1ml
Add DMSO just before use	

E199 Medium

10 x 199	50mls
29.5g/l Tryptose phosphate broth	50mls
80g/l Sodium bicarbonate	5mls
0.1M Glutamine	5mls
Distilled water	385mls

Add appropriate quantity of FBS when required

Foetal bovine serum (Growth 10%)	50mls
Foetal bovine serum (Maintenance 2%)	10mls

Store at 4°C

29.5g/l Tryptose phosphate both

Tryptose phosphate broth	29.5g
Distilled water	1000mls

Aliquot 20ml amounts. Autoclave 121°C for 20 minutes, pH7.3.

Store at room temperature

80g/l Sodium bicarbonate

Sodium hydrogen bicarbonate	80g
0.4% Phenol red (in distilled water)	5mls
Distilled water	1000mls
Filter 0.2nm, dispense into bijoux to the brim into metal cap.	
Store at room temperature	

0.1M Glutamine

Glutamine	29.2g
Distilled water	2000mls
Filter 0.2nm, aliquot into 20ml amounts, store at -20°C	
Shelf-life 4-6 months at -20°C, 3 weeks at 4°C	

Hanks (Balanced salt solution) HBSS

Hanks without sodium bicarbonate	10 litre pkt
Distilled water	10 litres
Place on stirrer to dissolve	
Sodium bicarbonate	3.5g
pH to 7.0-7.2, sterilise by filtration	
Aliquot into 200ml amounts	
Store at room temperature	

1% Nonidet P-40

NP40	1ml
1x Phosphate buffered saline	99mls
Store at 4°C	

0.4% Phenol Red

Phenol red	2g
1M Sodium hydroxide	6.25mls
Grind up in a mortar and pestle until dissolved	
Distilled water	118.75mls
Leave overnight pH 7.5-7.7	
Filter through Whatman's No 1 filter paper. Renew filter paper regularly.	
Sterilise by steaming for 2 hours.	
Store at 4°C	

10x Phosphate Buffered Saline (PBS)

Sodium chloride	800g
Potassium chloride	20g
Di-sodium hydrogen orthophosphate	115g
Potassium dihydrogen orthophosphate	20g
Distilled water	10 litres
Place on stirrer, dissolves readily	
Store at 4°C	

1M Sodium hydroxide

Sodium hydroxide	20g
Distilled water	500mls
Heat produced	
Store at room temperature	

0.2M Sodium carbonate

Sodium carbonate (anhydrous)	10.599g
Distilled water	500mls
Place on stirrer, dissolves readily	
Store 4°C	

0.2M Sodium hydrogen carbonate

Sodium hydrogen carbonate

8.4g

Distilled water

500mls

Place on stirrer, dissolves readily

Store 4°C

10x Phosphate buffered saline (PBS)

Sodium chloride

800g

Potassium chloride

20g

Disodium hydrogen orthophosphate

115g

Potassium dihydrogen orthophosphate

20g

Distilled water

10 litres

Place on stirrer, dissolves readily

Store at 4°C

0.1M Citric Acid

Citric acid

10.5g

Distilled water

500mls

Place on stirrer, dissolves readily

Store at 4°C

0.2M Di-sodium hydrogen orthophosphate

Di-sodium hydrogen orthophosphate (anhydrous)

14.19g

Distilled water

500mls

Place on stirrer, dissolves readily

Store at 4°C

2.5M Sulphuric Acid

Sulphuric acid

66.5mls

Distilled water

433.5mls

Use fume hood, ALWAYS add acid to water

Heat produced on addition of acid

Store at room temperature

Coating buffer: bicarbonate/carbonate pH9.6

0.2M sodium carbonate

7.9mls

0.2M sodium hydrogen carbonate

17.1mls

Distilled water

75mls

pH to 9.6 with 0.2M NaHCO₃. Make up fresh

Wash fluid: PBS/Tween 80/0.1 % BSA

10xPBS	100mls
Tween 80	0.5mls
Distilled water	900mls
Bovine serum albumin	1g
Place on a stirrer, dissolves readily	
Make up fresh	

Diluent: PBS/Tween 80/1 % BSA

10x PBS	20mls
Tween 80	0.1mls
Distilled water	180mls
Bovine serum albumin	2g
Place on a stirrer, dissolves readily	
Make up fresh	

Substrate: Orthophenylenediamine

0.1M Citric Acid	24.3mls
0.2M Di-sodium hydrogen orthophosphate	25.7mls
Distilled water	50mls
pH to 5.0 using either of above reagents	
Orthophenylene diamine (OPD)	0.08g
30% hydrogen peroxide	80µl

The substrate can be aliquoted into 20mls quantities prior to addition of the 30% H₂O₂ and frozen in the dark at -20°C.

Western blotting**4 x Laemmli gel buffer pH 8.8**

Tris	90.8g
Sodium dodecyl sulphate	2.0g
Distilled Water	500mls
Adjust pH to 8.8 with concentrated HCl	
Store at room temperature	

10% Ammonium persulphate

Ammonium persulphate	0.1g
Distilled Water	1ml
Store at 4°C up to one week	

2 x Stacking gel buffer

Tris	15.15g
Sodium dodecyl sulphate	1g
Distilled Water	500mls
Adjust pH to 6.8 with concentrated HCl	
Store at room temperature	

11 % Discontinuous gel

30% Acrylamide stock	11.3mls
4 x Laemmli gel buffer	10mls
Distilled Water	17.4mls
Sucrose (dissolves readily)	2.0g
Degas well, activate using the following TEMED	30µl
10% Ammonium persulphate	300µl
Make up fresh	

3 % Polyacrylamide stacking gel

30% Acrylamide stock	1ml
4 x Laemmli stacking gel buffer	5ml
Distilled Water	4ml
Degas well, activate using the following TEMED	10µl
10% Ammonium persulphate (sufficient for two gels - make up fresh)	50µl

Laemmli Electrode Buffer pH 8.3

Tris	15.14g
Glycine	72.09g
Sodium dodecyl sulphate	5.0g
Distilled Water	5000mls
pH should be 8.3	
Store at room temperature	

Transfer Buffer

Tris	5.8g
Glycine	29g
Methanol	400mls
Distilled Water	1600mls
Store at room temperature	

0.2% Ponceau S working dilution

2% Ponceau S stock solution	20mls
Distilled Water	180mls
Store at room temperature	

DAB 3,3' Diaminobenzidine (tetrahydrachloride)

DAB	5mg
Distilled Water	1ml
10mM Tris pH 7.0	10mls
30% w/v Hydrogen peroxide prepare fresh	200µl

1 x SDS gel loading buffer

40mM Tris HCl pH 6.8	90mls
Glycerol	10mls
Sodium dodecyl sulphate	2g
Bromophenol blue	0.1g
Mercaptoethanol	
Store at 4°C	

50mM TrisHCl pH 6.8

Tris	60.55g
Distilled water	200ml
pH to 6.8 using concentrated HCl	
Autoclave to sterilise and store at room temperature	

Radio immunoprecipitation**RIPA Lysis buffer**

1M Tris.Cl (pH 7.5)	100mls
Sodium Chloride	0.88g
Nonidet P40	1ml
Sodium dodecyl sulphate	1g
Deoxycholic acid(sodium salt)	0.5g
Place on stirrer to dissolve, dissolves readily	
Store at 4°C	

1M Tris.Cl pH 7.5

Tris	121.1g
Distilled Water	800mls
Concentrated Hydrochloric acid (HCl)	65mls
Adjust volume to 1 litre with distilled water and autoclave.	
Store at room temperature	

10mg/ml Aprotinin

Aprotinin	1mg
0.01M HEPES pH8.0	100µl
Aliquot into 5µl amounts, store at -20°C	
Before use make a 1/50 dilution in RIPA buffer(5µl + 245µl RIPA buffer),	
add 2.5µl of diluted Aprotinin per petri dish	
Effective concentration 1-2µg/ml. The stock solution should be stored in small aliquots at -20°C. Each aliquot should be discarded after use.	

10mg/ml Phenylmethylsulfonyl Fluoride (PMSF)

PMSF(10mg/ml)	10mg
Isopropanol	1ml
Aliquot into 50µl. Use as stored, 2.5µl per petri dish. Store at -20°C	
Effective concentration 100µg/ml	

PMSF is inactivated in aqueous solutions. The rate of inactivation increases with pH and is faster at 25°C than at 4°C. The half-life of a 20µM aqueous solution of PMSF is about 35 minutes at pH8.0. This means that solutions of PMSF can be safely discarded after they have been rendered alkaline(pH>8.6) and stored for several hours at room temperature.

15 % SDS PAGE gel

30% Acrylamide stock	30ml
4 x Laemmli gel buffer pH8.8	15ml
Sucrose	3g
Distilled Water	14.5ml

Degas well, activate using the following

TEMED	37.5µl
10% Ammonium persulphate	375µl
60mls sufficient to pour 1gel.	

3% SDS PAGE gel (10mls)

*30% Acrylamide stock	1ml
4 Laemmli stacking buffer pH6.8	5ml
Distilled water	4ml

Degas well, activate using the following

*TEMED	10µl
*10% Ammonium persulphate	50µl
10mls sufficient to pour 1 gel	

0.01M HEPES pH8.0

HEPES	0.566g
Distilled Water	200mls
pH to 8.0 using 1M Sodium hydroxide	
Store at 4°C	

1 x RPMI 1640 Methionine free

1 x RPMI 1640	20ml
100x Arginine	0.2ml
100x Cysteine	0.2ml
100x Leucine	0.2ml
100x Inositol	0.2ml
100x Glucose	0.2ml
0.1M Glutamine	0.2ml
Store at 4°C	

10% Ammonium Persulphate

Ammonium persulphate	0.1g
Distilled water	1ml
Store at 4°C up to one week	

4 x Laemmli gel buffer

Tris	90.8g
Sodium dodecyl sulphate	2.0g
Distilled water	500mls
Adjust pH to 8.8 with concentrated HCl	
Store at room temperature	

2 x Stacking gel buffer

Tris	15.15g
Sodium dodecyl sulphate	1g
Distilled water	500mls
Adjust pH to 6.8 with concentrated HCl	
Store at room temperature	

Laemmli Electrode Buffer

Tris	15.14g
Glycine	72.09g
Sodium dodecyl sulphate	5.0g
Distilled water	5000mls
pH should be 8.3	
Store at room temperature	

1 x SDS gel loading buffer

50mM Tris HCl pH6.8	90ml
Glycerol	10ml
Sodium dodecyl sulphate	2g
Bromophenol blue	0.1g
2-Mercaptoethanol	5mls
Store at 4°C	

50mM Tris HCl pH6.8

Tris	60.55g
Distilled water	200ml
pH to 6.8 using concentrated HCl	
Autoclave to sterilise	
Store at room temperature	

1M Sodium Hydroxide

Sodium hydroxide	20g
Distilled water	500ml
Heat may be produced	
Store at room temperature	

0.1M Glutamine

Glutamine

29.2g

Distilled water

2000mls

Sterilise through 0.2nm filter

Store at -20°C

Developer

Developer

1 container

Tap water

5 litres

Aliquot into 500ml

Store in the dark at room temperature

Fixative

Kodak Unifix

1 litre

Tap water

4 litres

Aliquot into 1 ml Store in the dark at room temperature

APPENDIX
A WORKED EXAMPLE OF COMPETITION
ELISA RESULTS FROM SECTION 6.2.2

Competition ELISA OD for Table 6.2b

Competition between non-conjugated 9C3 and 6E2 HRP mabs at 1/2000 dilution	=	[0.87] = <0	-	[0.92]
Competition between non-conjugated 9C3 and 7C9 HRP mabs at 1/2000 dilution	=	[0.21] = 0	-	[0.21]
Competition between non-conjugated 9C3 and 6E8 HRP mabs at 1/2000 dilution	=	[2.13] = <0	-	[2.26]
Competition between non-conjugated 9C3 and 8B6 HRP mabs at 1/2000 dilution	=	[0.25] = <0	-	[0.27]
Competition between non-conjugated 9C3 and 9C3 HRP mabs at 1/2000 dilution	=	[0.76] = 0.70	-	[0.06]
Competition between non-conjugated 9C3 and 10E6 HRP mabs at 1/2000 dilution	=	[0.06] = 0	-	[0.06]
Competition between non-conjugated 9C3 and 13C10 HRP mabs at 1/2000 dilution	=	[0.23] = 0.22	-	[0.01]